BM

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12N 5/08 | A2

(11) International Publication Number: **WO 00/44882**

(43) International Publication Date: 3 August 2000 (03.08.00)

(21) International Application Number: PCT/US00/02208

(22) International Filing Date: 28 January 2000 (28.01.00)

(30) Priority Data:
09/240,029 29 January 1999 (29.01.99) US

(71) Applicant *(for all designated States except US)*: ZEN-BIO, INC. [US/US]; Suite 102, 3200 Chapel Hill-Nelson Boulevard, Research Triangle Park, NC 27709 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: HALVORSEN, Yuan-Di, Chang [-/US]; 5128 Salem Ridge Road, Holly Springs, NC 27540 (US). WILKISON, William, O. [US/US]; 9706 Gallop Lane, Bahama, NC 27503 (US).

(74) Agents: SALE, Elaine, T. et al.; Alston & Bird LLP, P.O. Drawer 34009, Charlotte, NC 28234-4009 (US).

(81) Designated States: CN, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: METHODS AND COMPOSITIONS FOR THE DIFFERENTIATION OF HUMAN PREADIPOCYTES INTO ADIPOCYTES

(57) Abstract

The present invention provides methods and compositions for the consistent and quantitative differentiation of human preadipocytes isolated from adipose tissue into adipocytes bearing biochemical, genetic, and physiological characteristics similar to that observed in isolated primary adipocytes. The methods of the invention comprise incubating isolated human preadipocytes, plated at least about 25,000 cells/cm^2, in a medium containing, glucose, a cyclic AMP inducer such as isobutylmethylxanthine or forskolin, a glucocorticoid or glucocorticoid analogue, insulin or an insulin analogue and a PPAR$_\gamma$ agonist or a RXR agonist. Also provided are methods for preparing three dimensional biomatrices containing adipocytes differentiated by the methods of the invention. The compositions of the invention include human adipocytes differentiated by the methods of the invention, three-dimensional matrices of adipocytes, and transfected adipocytes. The methods and compositions have use in the drug discovery of compounds having relevance to the disease states of diabetes, obesity, and cardiovascular disease and in the studies of these diseases, and in the grafting of fat tissue.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

METHODS AND COMPOSITIONS FOR THE DIFFERENTIATION OF HUMAN PREADIPOCYTES INTO ADIPOCYTES

FIELD OF THE INVENTION

The invention is drawn to the field of adipocyte biology. Methods and compositions are provided for the differentiation of human preadipocytes into adipocytes.

BACKGROUND OF THE INVENTION

Non-insulin dependent diabetes mellitus (NIDDM) afflicts 4-5 million Americans every year. NIDDM is treated predominately with insulin. However, insulin is not convenient to use in that it must be injected 2-4 times per day and must be stored properly to prevent loss of efficacy. Other drugs used to treat NIDDM include troglitazone (Rezulin™), a $PPAR_{\gamma}$ agonist, Glucophage™ and sulfonylureas. Unfortunately, there are safety concerns related to the use of these drugs. The identification of safe, effective, orally available drugs for the treatment of NIDDM would greatly enhance the quality of life of patients who suffer from this disease. However, studies to find such molecules have been hampered by a lack of reproducible human *in vitro* cell systems.

Approximately 20-25% of Americans are obese and therefore at increased risk for developing NIDDM, hypertension, and cardiovascular disease. The causes of NIDDM and obesity are often related to defects or problems with adipose tissue. Adipocytes play a critical role in lipid storage and metabolism. Adipocytes also act as endocrine cells to

influence physiological parameters such as insulin sensitivity and body weight (Flier, *et al., Cell,* (1995) 80:15-18). For example, the *ob* gene encodes leptin, an adipocyte-secreted endocrine factor (Zhang, *et al., Nature* (1994) 372:425-432). Leptin has been shown to reduce body weight and blood glucose in obese, diabetic rodents (Pelleymounter, *et al., Science,* (1995) 269:540-543).

Several adipocyte-specific enzymes and receptors have been shown to be important targets for anti-obesity and anti-diabetic drug discovery. For example, agonists of the $\beta 3$ adrenergic receptor, which is found predominantly in the adipose tissue in man (Arner, *et al., New England Journal of Medicine,* (1995) 333:382-383), have anti-obesity and anti-diabetic properties in rodents and are currently in phase II/III trials in man. The thiazolidinedione class of compounds (TZDs), including troglitazone and ciglitazone, has been shown to improve insulin sensitivity and thereby reduce hyperglycemia and hyperlipidemia conditions in rodents and in humans (Saltiel, *et al., Diabetes,* (1996) 45:1661-1669; Sreenan, *et al., American Journal Physiol,* (1996) 271:E742-E747; Nolan, *et al., New England Journal of Medicine,* (1994) 331:1188-1193. Troglitazone (Rezulin™) is approved for use in the U.S. and Japan. Many TZDs, including troglitazone and ciglitazone, are potent activators of Peroxisome Proliferator Activated Receptor gamma ($PPAR_\gamma$), a member of the nuclear receptor family of transcription factors (Tontonoz , *et al., Cell,* (1994) 79:1147-1156; Lehmann, *et al., Journal of Biological Chemistry,* (1995) 270:12953-12955). $PPAR_\gamma$ is a key regulator of adipocyte differentiation and is most abundant in adipose tissue.

Animal adipocyte studies have been facilitated by the availability of a number of immortalized preadipocyte cell lines such as the 3T3-L1 mouse fibroblast line (Green, *et al., Cell,* (1974) 1:113-116), which upon proper treatment, will differentiate into adipocytes. These cells have many of the same properties as isolated primary adipocytes. However, recent published reports show that human adipose tissue and adipocytes exhibit significant differences from rodent cells with respect to factors affecting insulin resistance. For example, TNF_α appears to be regulated differently in human adipose tissue than in rodent adipose tissue (Hotamisligil, *et al., Journal of Clin Invest,* (1995) 95:2409-2415). Consequently, studies on human adipocytes and adipose metabolism have

been hampered by the lack of a preadipocyte cell culture that can be reproducibly induced to differentiate into adipocytes at high efficiency.

Current protocols for differentiating isolated human preadipocytes result in differentiation frequencies of 5-80%. In these systems, the preadipocyte cell component in human adipose tissue (the so-called "stromal vascular fraction" or SVF) can be isolated using collagenase treatment (Rodbell, *Journal of Biol Chem.*, (1967) 242:5744-5750; Rodbell, *et al., Meth Enzymol*, (1974) 31:103-14). The isolated human preadipocytes can then be driven to differentiate into adipocytes by a variety of chemical treatments. For example, Hauner's laboratory (Hauner, *et al., Journal Clin Invest.*, (1989) 34:1663-1670) has shown that human preadipocytes can be induced to differentiate in serum-free medium containing 0.2 nM triiodothyronine, 0.5 μM insulin and 0.1 μM glucocorticoid (cortisol, dexamethasone or aldosterone). Under these conditions, differentiation of 5-70% of the preadipocytes was achieved. The percentage of differentiated cells was related to the age of the subject from which the cells were obtained. These investigators claim they can achieve anywhere from 5-70% complete differentiation within 20 days as determined by a variety of biochemical markers. Similarly, O'Rahilly's laboratory (Digby, *et al., Diabetes,* (1998) 5:138-141) have shown that when the above serum-free medium is supplemented with a TZD such as BRL 49653, differentiation of 20% of omental preadipocytes and 50-80% of subcutaneous and perirenal preadipocytes is achieved.

A method of differentiating human preadipocytes to adipocytes at higher frequency in a shorter period of time and with greater consistency would aid in the study of obesity and diabetes. The present invention provides methods and compositions for the consistent differentiation of 90-95% of human preadipocytes into adipocytes, in both 2 dimensional culture and in three-dimensional biocompatible matrices.

SUMMARY OF THE INVENTION

The present invention provides methods and compositions for the consistent and quantitative differentiation of human preadipocytes isolated from adipose tissue into adipocytes bearing biochemical, genetic, and physiological characteristics similar to that

observed in isolated primary adipocytes. The methods of the invention comprise incubating isolated human preadipocytes, plated at least about 25,000 cells/cm^2, in a medium containing, glucose, a cyclic AMP inducer such as isobutylmethylxanthine or forskolin, a glucocorticoid or glucocorticoid analogue, insulin or an insulin analogue and a PPAR$_\gamma$ agonist or a RXR agonist, in the presence or absence of a three-dimensional biocompatible matrix. The compositions of the invention include human adipocytes differentiated by the methods of the invention and transfected adipocytes.

The present invention also provides methods for determining the ability of a compound to affect the differentiation of human preadipocytes to adipocytes, for determining the ability of a compound to act as a PPAR$_\gamma$ antagonist, glucocorticoid, glucocorticoid analogue or an insulin analogue, for transfecting cultured human adipocytes, and as a means to identify novel polypeptides secreted from human adipocytes into the conditioned medium. The methods and compositions have use in the drug discovery of compounds having relevance to the disease states of diabetes, obesity, and cardiovascular disease and in the studies of these diseases, and in the grafting of adipose tissue.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows normal human preadipocytes isolated from subcutaneous adipose tissue (Panel A) and human adipocytes differentiated from preadipocytes using the methods described herein (Panel B).

Figure 2 is a graphical representation of the optical density representing staining with Oil Red O of adipocytes differentiated by the methods of the invention in comparison to the Oil Red O staining of preadipocytes.

Figure 3 is a graphical representation of the dose response relationship of BRL49653 in differentiating human preadipocytes.

Figure 4 is a graphical representation of the differentiation of human preadipocytes in response to increasing dexamethasone concentrations.

Figure 5 is a graphical representation of the inhibition of differentiation of human preadipocytes by novel compounds.

Figure 6 is a graphical representation of the lipolytic response to isoproterenol of human adipocytes differentiated from preadipocytes isolated from five individuals.

Figure 7 is a graphical representation of the β-galactosidase expression at 4, 7 and 14 days post-transfection for human differentiated adipocytes transfected with various amounts of pCMV-βgal in the presence of Effectene.

Figure 8 is an autoradiograph of total human preadipocyte and differentiated adipocyte secreted proteins separated by isoelectric focusing and two-dimensional gel electrophoresis.

Figure 9 shows the detection of the human leptin, an adipocyte-derived protein hormone, by enzyme linked immunoassay (ELISA) (A) and by immunoblotting (B) in the conditioned medium of human adipose tissue derived stromal cells under control (dots) and adipocyte inducing conditions (insulin, stipple).

Figure 10 shows adipocytes differentiated from human adipose tissue-derived stromal cells transduced using an adenoviral/CMV promoter-beta galactosidase expression vector.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods and compositions for the consistent and quantitative differentiation of human preadipocytes isolated from adipose tissue into adipocytes bearing biochemical, genetic, and physiological characteristics similar to that observed in isolated primary adipocytes. The prior art methods and compositions produce inconsistent differentiation frequencies of 5-80%. See Hauner *et al.* and Digby *et al., supra.* In contrast, the methods of the invention reproducibly achieve 90-95% differentiation of cultured human preadipocytes into adipocytes and shorten the culture time from 21 to 12 days.

Thus, in one aspect, the invention provides a method for differentiating human preadipocytes into adipocytes, comprising:

a) plating isolated human preadipocytes at a density of about 25,000 to 30,000 cells/cm^2 in a preadipocyte medium comprising a cell culture medium having or supplemented with 1.0-4.5 g/liter glucose and 0-15% fetal serum;

b) incubating said cells at about 37°C for about 4-48 hours until said cells are about 95-100% confluent;

c) replacing said preadipocyte medium with a differentiation medium comprising a cell culture medium having or supplemented with 1.0-4.5 g/liter glucose, 1-15% fetal serum; a cyclic AMP inducer; 100 nM to 1 μM insulin, or an equivalent amount of an insulin analogue; 16 nM to 1 μM of a glucocorticoid; and a concentration of a $PPAR_\gamma$ agonist or RXR agonist effective to stimulate differentiation of human preadipocytes;

d) incubating said cells at about 37°C for about 2-4 days;

e) replacing said differentiation medium with an adipocyte medium comprising a cell culture medium having or supplemented with 1.0-4.5 g/liter glucose; 0-15% fetal serum; 100 nM to 1 μM insulin, or an equivalent amount of an insulin analogue; 16 nM to 1 μM of a glucocorticoid; wherein said adipocyte medium contains neither an effective amount of a cAMP inducer nor a concentration of a $PPAR_\gamma$ agonist or RXR agonist effective to stimulate differentiation of human preadipocytes; and

f) incubating said cells at about 37°C for about 1-2 weeks and refeeding said cells with said adipocyte medium at least every 3-4 days;

wherein said preadipocyte medium, said differentiation medium and said adipocyte medium are maintained at a pH of about 7.0 to 7.6 when in contact with said cells.

By "preadipocyte" is meant cells that could be isolated from a stromal vascular fraction prepared from adipose tissue, and that have the potential to differentiate into adipocytes. However, the preadipocytes used in the methods of the invention may be isolated by any means known to those skilled in the art. Preferably, the preadipocytes are isolated from a stromal vascular fraction prepared from adipose tissue.

A variety of cell culture media, known to those skilled in the art, are useful in the methods of the invention. Examples of such media (all without serum or having had the serum removed) include Minimum Essential Medium Eagle, ADC-1, LPM (Bovine Serum Albumin-free), F10(HAM), F12 (HAM), DCCM1, DCCM2, RPMI 1640, BGJ Medium (with and without Fitton-Jackson Modification), Basal Medium Eagle (BME-

with the addition of Earle's salt base), Dulbecco's Modified Eagle Medium (DMEM-without serum), Yamane, IMEM-20, Glasgow Modification Eagle Medium (GMEM), Leibovitz L-15 Medium, McCoy's 5A Medium, Medium M199 (M199E- with Earle's sale base), Medium M199 (M199H- with Hank's salt base), Minimum Essential Medium Eagle (MEM-E- with Earle's salt base), Minimum Essential Medium Eagle (MEM-H- with Hank's salt base) and Minimum Essential Medium Eagle (MEM-NAA- with non-essential amino acids), among numerous others, including medium 199, CMRL 1415, CMRL 1969, CMRL 1066, NCTC 135, MB 75261, MAB 8713, DM 145, Williams' G, Neuman & Tytell, Higuchi, MCDB 301, MCDB 202, MCDB 501, MCDB 401, MCDB 411, MDBC 153. Preferably the medium is Dulbecco's Modified Eagle Medium /Ham's F-10 Nutrient Broth (1:1 v/v) (See Ham, *Exp Cell Res.*, (1963) 29: 515; Morton, *In Vitro*, (1970) 6:89-108; Dulbecco, *et al., Virology* (1959) 8:396; Smith, *et al., Virology,* (1960) 12:185) or Earl's medium (Earle, 1943 *JNCI* 4:165-169). These and other useful serum-free nutrient media are available from GIBCO, Grand Island, N.Y., USA and Biological Industries, Bet HaEmek, Israel, among others. A number of these media are summarized in Methods in Enzymology, Volume LVIII, "Cell Culture", pp. 62-72, edited by William B. Jakoby and Ira H. Pastan, published by Academic Press, Inc.

Additional compounds may be included or added to the medium. For example, antibiotics, such as penicillin, streptomycin and fungizone are useful additives to the media of the invention. Also, the addition to the media of about 0.5-100 μM biotin, preferably about 10-60 μM biotin, more preferably about 30-35 μM biotin, most preferably about 33 μM biotin; and about 0.5-100 μM pantothenate, preferably 5-50 μM pantothenate, more preferably 15-25 μM pantothenate, most preferably about 17 μM pantothenate, may be useful in promoting cell growth.

The pH of the medium must be maintained at a pH of about 7.0 to 7.6 during use, either through the inclusion of a biological buffer or by adjusting the CO_2 content in the atmosphere of the incubator. A "biological buffer" is a mixture of a weak acid and its conjugate base that tends to resist changes in pH when small amounts of acid or base are added. Typical biological buffers include, but are not limited to, phosphate and carbonate buffering systems. Preferably, the medium will be maintained at a pH of about 7.2-7.5,

more preferably 7.3 to 7.5, most preferably at a pH of about 7.4. Preferably the medium is buffered by about 15 mM $NaHCO_3$ and about 15 mM HEPES, at a pH of about 7.3-7.5, preferably at a pH of about 7.4.

The preferred fetal serum is mammalian fetal serum, preferably fetal bovine serum. Fetal bovine serum (FBS) can be added to the cell culture medium at a concentration of about 1 to 15%, preferably about 1-10%, most preferably about 3-10%. A concentration of at least about 3% FBS is necessary to ensure that the cells attach firmly to the tissue culture container.

By "cyclic AMP inducer" is meant any compound that, in an effective concentration, increases the intracellular concentration of cAMP in a preadipocyte or adipocyte by at least 2%, preferably by at least 5%, more preferably by at least 10%, most preferably by at least 20%. Methods for measuring intracellular cAMP levels are known to those skilled in the art. Preferred cyclic AMP inducers include isobutylmethylxanthine and forskolin. Preferably, the cyclic AMP inducer is 0.2 to 5 mM isobutylmethylxanthine, more preferably, 0.5 to 0.5 mM isobutylmethylxanthine.

By "insulin" is meant any naturally occurring or recombinant mammalian insulin. Preferably the insulin is naturally occurring or recombinant human insulin. By "equivalent amount of an insulin analogue" is meant an amount of a compound that stimulates the differentiation of human stromal vascular cells to adipocytes, using the methods and media of the invention, to the same degree as does 100 nM to 1 μM human insulin. Such insulin analogues may or may not be structurally related to insulin and may be naturally occurring synthetic or recombinant. Preferred insulin analogues are insulin-like growth factor I and insulin-like growth factor II. The percentage of preadipocytes that differentiate into adipocytes may be quantitatively determined by a variety of methods known to those skilled in the art. A preferred method is by Oil Red O staining (Green, *supra*).

By "glucocorticoid" is meant any steroid or steroid-like compounds and functional derivatives thereof, which is capable of inducing the differentiation of preadipocytes into adipocytes, using the media and methods of the invention. Preferably, the glucocorticoid is dexamethasone, hydrocortisone or cortisol. A physiological concentration of glucocorticoid is used. Preferably the concentration of glucocorticoid is

about 16 nM to 1 μM, more preferably about 100 nM to 1 μM, most preferably the concentration of glucocorticoid is about 1 μM.

By "PPAR$_\gamma$ agonist" is meant a compound capable of activating the peroxisome proliferator-activated receptor gamma (PPAR$_\gamma$) by at least 10% over background. PPAR$_\gamma$ activation may be determined by the method described by Lehmann *et al. supra*. Preferably the PPAR$_\gamma$ agonist is a thiazolidinedione of a derivative or analogue thereof. More preferably the PPAR$_\gamma$ agonist is BRL 49653, pioglitizone, troglitazone, darglitazone, ciglitazone, AD5075, AD5080, AD4742 or AD4743. Most preferably the PPAR$_\gamma$ agonist is BRL 49653 or troglitazone and the concentration of BRL 49653 or troglitazone is about 0.5-1.0 μM. More preferably the concentration of BRL 49653 or troglitazone is about 0.8-1.0 μM, most preferably the concentration is about 1.0 μM.

By "RXR agonist" is meant a compound capable of activating a retinoic acid X receptor (RXR) by at least 10% over background. RXR activation may be determined by the method described by Heyman *et al.* (1992 *Cell* 68:397-406). Preferably, the RXR agonist is LG1069 or *cis*-retinoic acid. Preferably the concentration of LG1069 or *cis*-retinoic acid is about 100 nM to 10 μM, most preferably the concentration is about 1 μM.

By "effective to stimulate the differentiation of human preadipocytes" is meant having at least about the same effect on the ability to stimulate differentiation of human preadipocytes to adipocytes using to the methods of the invention, as does 0.5-1.0 μM BRL 49653. The percentage of preadipocytes that differentiate into adipocytes may be quantitatively determined by a variety of methods known to those skilled in the art. A preferred method is by Oil Red O staining.

Human adipose tissue from a variety of sources may be processed to produce preadipocytes for the generation of adipocytes. The adipose tissue may be from subcutaneous or perirenal sites. Preferably the adipose tissue is subcutaneous. Liposuction surgery or penniculectomy may provide subcutaneous adipose tissue.

Preadipocytes may be isolated from adipose tissue by a variety of methods known to those in the art. Preferably the preadipocytes are isolated from the stromal vascular fraction by the method of Rodbell (1974), *supra*.

When initially plating preadipocytes in preadipocyte medium (step a), the cells must be plated at a density of 25,000-40,000 cells/cm^2. Preferably the cell density is 25,000-30,000 cells/cm^2. Lower density plating of preadipocytes results in an overall lower differentiation percentage. When plated at a density of 25,000 cells/cm^2 the preadipocytes are usually confluent after overnight incubation. If cells are not fully confluent at this point, they may be incubated for up to another 24 hours prior to refeeding with differentiation medium. Longer incubations will result in a lower differentiation percentage.

Once the cells have been exposed to differentiation media (step c), they are susceptible to detaching from the plate if the media is either completely removed or quickly added. When the differentiation or adipocyte medium is changed, care should be taken to leave a layer of medium over the cell surface (approximately 30-50 μl/well for a 96-well plate).

Formation of oil droplets, a characteristic of adipocytes, will occur approximately four days after differentiation medium is added. However, there will be some variation related to inter-patient variability and the site from which the preadipocytes were isolated. In general, at least 90%, typically 90-95% of cells, will differentiate under the above conditions.

A further object of the invention is to provide methods for the identification and study of compounds that enhance preadipocyte differentiation to adipocytes. It has been demonstrated that many compounds that enhance the differentiation of preadipocytes to adipocytes play a role in the treatment of diabetes mellitus. For example, compounds such as insulin, glucocorticoids, and peroxisome proliferator activated receptor gamma ($PPAR_\gamma$) activators effect glucose clearance and mediate the differentiation of preadipocytes to adipocytes. Insulin and Rezulin™ (a $PPAR_\gamma$ activator) are currently used to treat diabetes in the U.S. Compounds that affect human adipocyte differentiation or lipogenesis may be useful in the treatment of diabetes and obesity. Such compounds include $PPAR_\gamma$ agonists, RXR agonists and insulin analogues.

Accordingly, a method is provided for determining the ability of a compound to affect the differentiation of preadipocytes to adipocytes, comprising:

a) plating isolated human preadipocytes at a density of about 25,000 to 30,000 cells/cm^2 in a preadipocyte medium comprising a cell culture medium having or supplemented with 1.0-4.5 g/liter glucose and 0-15% fetal serum;

b) incubating said cells at about 37°C for about 4-48 hours until said cells are about 95-100% confluent;

c) replacing said preadipocyte medium with a differentiation medium comprising a cell culture medium having or supplemented with 1.0-4.5 g/liter glucose, 1-15% fetal serum; a cyclic AMP inducer; 100 nM to 1 μM insulin, or an equivalent amount of an insulin analogue; 16 nM to 1 μM of a glucocorticoid; a concentration of a PPAR$_\gamma$ agonist or RXR agonist effective to stimulate differentiation of human preadipocytes; and said compound in an appropriate vehicle or vehicle alone;

d) incubating said cells at about 37°C for about 2-4 days;

e) replacing said differentiation medium with an adipocyte medium comprising a cell culture medium having or supplemented with 1.0-4.5 g/liter glucose; 0-15% fetal serum; 100 nM to 1 μM insulin, or an equivalent amount of an insulin analogue; 16 nM to 1 μM of a glucocorticoid; wherein said adipocyte medium contains neither an effective amount of a cAMP inducer nor a concentration of a PPAR$_\gamma$ agonist or RXR agonist effective to stimulate differentiation of human preadipocytes; and

f) incubating said cells at about 37°C for about 1-2 weeks and refeeding said cells with said adipocyte medium at least every 3-4 days; and

g) determining the number or percentage of differentiated cells in the cells from step (f) fed at step (c) with said differentiation medium containing said compound;

h) determining the number or percentage of differentiated cells in the cells from step (f) fed at step (c) with said differentiation medium containing said vehicle alone; and

i) comparing the number or percentage of differentiated cells from steps (g) and (h);

wherein said preadipocyte medium, said differentiation medium and said adipocyte medium are maintained at a pH of about 7.0 to 7.6 when in contact with said cells.

Any compound may be tested for its ability to affect the differentiation of preadipocytes to adipocytes. Compounds of particular interest include agonists of $PPAR_\gamma$ and/or RXR receptors, such as TZDs. Appropriate vehicles compatible with the compound to be tested are known to those skilled in the art and may be found in a current edition of Remington's Pharmaceutical Sciences. Examples of appropriate vehicles include, but are not limited to, DMSO, DMF or ethanol solutions and a variety of aqueous buffers.

A variety of methods known to those skilled in the art may be used to determine the percentage of differentiated cells. Examples of such methods include those that assess biochemical or morphological characteristics, such as lipid deposits and adipocyte-specific proteins or mRNAs. In a preferred method, the cells are fixed in formalin and stained with Oil Red O dye.

In another embodiment, the invention provides a method for determining the ability of a compound to act as a $PPAR_\gamma$ antagonist. Thus, the invention provides a method for determining the ability of a compound to act as a $PPAR_\gamma$ antagonist, comprising:

a) plating isolated human preadipocytes at a density of about 25,000 to 30,000 cells/cm^2 in a preadipocyte medium comprising a cell culture medium having or supplemented with 1.0-4.5 g/liter glucose and 0-15% fetal serum;

b) incubating said cells at about 37°C for about 4-48 hours until said cells are about 95-100% confluent;

c) replacing said preadipocyte medium with a differentiation medium comprising a cell culture medium having or supplemented with 1.0-4.5 g/liter glucose, 1-15% fetal serum; a cyclic AMP inducer; 100 nM to 1 μM insulin, or an equivalent amount of an insulin analogue; 16 nM to 1 μM of a glucocorticoid; and a concentration of a $PPAR_\gamma$ agonist effective to stimulate half-maximal differentiation of a human preadipocytes; and said compound in an appropriate vehicle or vehicle alone;

d) Incubating said cells at about 37°C for about 2-4 days;

e) replacing said differentiation medium with an adipocyte medium comprising a cell culture medium having or supplemented with 1.0-4.5 g/liter glucose; 0-15% fetal serum; 100 nM to 1 μM insulin, or an equivalent amount of an insulin analogue; 16 nM to 1 μM of a glucocorticoid; wherein said adipocyte medium contains neither an effective amount of a cAMP inducer nor a concentration of a $PPAR_\gamma$ agonist or RXR agonist effective to stimulate differentiation of human preadipocytes; and

f) Incubating said cells at about 37°C for about one week and refeeding said cells at least once with the supplemented medium from step (e); and

g) Determining the number or percentage of differentiated cells in the cells from step (f) fed at step (c) with medium containing said compound;

h) Determining the number or percentage of differentiated cells in the cells from step (f) fed at step (c) with medium containing said vehicle alone; and

i) Comparing the number or percentage of differentiated cells from steps (g) and (h);

wherein said preadipocyte medium, said differentiation medium and said adipocyte medium are maintained at a pH of about 7.0 to 7.6 when in contact with said cells.

A variety of $PPAR_\gamma$ agonists may be used. Preferentially, the $PPAR_\gamma$ agonist is a TZD. Preferably the $PPAR_\gamma$ agonist is BRL 49653 at a concentration of about 1-500 nM, preferably about 10-100 nM, most preferably about 50 nM.

In yet another embodiment the invention provides a method for determining the ability of a compound to act as a glucocorticoid or glucocorticoid analogue, comprising:

a) plating isolated human preadipocyte cells at a density of about 25,000 to 40,000 cells/cm^2 in a preadipocyte medium comprising a defined cell culture medium having or supplemented with 1.0-4.5 g/liter glucose;

b) incubating said cells at about 37°C for about 4-24 hours until said cells are about 95-100% confluent;

c) replacing said preadipocyte medium with a differentiation medium comprising a defined cell culture medium having or supplemented with 1.0-4.5 g/liter

glucose; a cyclic AMP inducer; 100 nM to 1 μM insulin, or an equivalent amount of an insulin analogue; and a concentration of a $PPAR_\gamma$ agonist or RXR agonist effective to stimulate differentiation of a human preadipocytes; and said compound in an appropriate vehicle or vehicle alone;

d) incubating said cells at about 37°C for about 2-4 days;

e) replacing said differentiation medium with an adipocyte medium comprising a defined cell culture medium having or supplemented with 1.0-4.5 g/liter glucose; 100 nM to 1 μM insulin, or an equivalent amount of an insulin analogue; and said compound in an appropriate vehicle or vehicle alone;

f) incubating said cells at about 37°C for about 1-2 weeks and refeeding said cells at least every 3-4 days with said adipocyte medium; and

g) determining the number or percentage of differentiated cells in the cells from step (f) fed at step (c) with medium containing said compound;

h) determining the number or percentage of differentiated cells in the cells from step (f) fed at step (c) with medium containing said vehicle alone; and

i) comparing the number or percentage of differentiated cells from steps (g) and (h);

wherein said preadipocyte medium, said differentiation medium and said adipocyte medium are maintained at a pH of about 7.0 to 7.6 when in contact with said cells.

In still another embodiment the invention provides a method for determining the ability of a compound to act as an insulin analogue, comprising:

a) plating isolated human preadipocytes at a density of about 25,000 to 30,000 cells/cm^2 in a preadipocyte medium comprising a cell culture medium having or supplemented with 1.0-4.5 g/liter glucose and 0-15% fetal serum;

b) incubating said cells at about 37°C for about 4-48 hours until said cells are about 95-100% confluent;

c) replacing said preadipocyte medium with a differentiation medium comprising a cell culture medium having or supplemented with 1.0-4.5 g/liter glucose, 1-15% fetal serum; a cyclic AMP inducer; 16 nM to 1 μM of a glucocorticoid; and a

concentration of a $PPAR_\gamma$ agonist or RXR agonist effective to stimulate differentiation of a human preadipocytes; and said compound in an appropriate vehicle or vehicle alone;

d) incubating said cells at about 37°C for about 2-4 days;

e) replacing said differentiation medium with an adipocyte medium comprising a cell culture medium having or supplemented with 1.0-4.5 g/liter glucose; 0-15% fetal serum; 16 nM to 1 μM of a glucocorticoid; and said compound in an appropriate vehicle or vehicle alone; wherein said adipocyte medium contains neither an effective amount of a cAMP inducer nor a concentration of a $PPAR_\gamma$ agonist or RXR agonist effective to stimulate differentiation of human preadipocytes;

f) incubating said cells at about 37°C for about 1-2 weeks and refeeding said cells at least every 3-4 days with said adipocyte medium; and

g) determining the number or percentage of differentiated cells in the cells from step (f) fed at step (c) with medium containing said compound;

h) determining the number or percentage of differentiated cells in the cells from step (f) fed at step (c) with medium containing said vehicle alone; and

i) comparing the number or percentage of differentiated cells from steps (g) and (h);

wherein said preadipocyte medium, said differentiation medium and said adipocyte medium are maintained at a pH of about 7.0 to 7.6 when in contact with said cells.

A number of studies have suggested that compounds that can induce lipolysis in adipocytes may have utility in treating obesity or cardiovascular disease. The adipocytes differentiated according to the methods of the invention are responsive to lipolytic agents such as norepinephrine and isoproterenol, a β-adrenoreceptor agonist. When stimulated by lipolytic compounds, triglycerides in adipocytes are converted to glycerol and fatty acids. The glycerol is then released from the cells to the medium. Measurement of glycerol in the conditioned medium is an indication of the response of adipocytes to a lipolytic agent. Accordingly, the mature differentiated adipocytes of the invention may be used to identify and study compounds affecting lipolysis. The ability of a compound to stimulate lipolysis in human adipocytes may also be investigated using this system.

Upon full differentiation of the adipocytes, compounds can be added to the cell system to determine their ability to stimulate lipolysis via glycerol production. Glycerol concentration may then be determined using standard methods (Barham, *et al., Analyst,* (1972) 97:142).

The ability to introduce exogenous DNA transiently into cultured human adipocytes will enable the investigator to analyze production of proteins in the adipocyte, characterize promoter regions of adipocyte-selective genes, and identify compounds that affect the expression and regulation of both these described systems. In systems used prior to the instant invention, the inability to obtain an enriched (>90%) population of differentiated adipocytes hampered the use of transfecting exogenous DNA for the purposes of analysis of gene expression. The mixture of preadipocytes and adipocytes in varying proportions precludes the precise analysis of gene regulation because of the existence of two cell types. The availability of a relatively pure population of adipocytes allows for the precise and careful analysis of gene expression via the introduction of exogenous DNA. Thus, in still another embodiment, the invention provides transfected adipocytes differentiated from preadipocytes by the methods of the invention. A variety of transfection protocols using agents known to those skilled in the art, such as calcium phosphate (Wigler, *et al., Cell,* (1977) 11:223-231); lipofectin; (Felgner, *et al., Proc Natl Acad Sci USA*, (1987) 87:7413-7417); or Effectene may be used. Alternatively, viral particles that can infect preadipocytes, for example, adenovirus, may be used to introduce DNA into the stromal cells similarly to the protocols described (Becker, *et al., Meth Cell Biol,* (1994) 43:161-189; Meunier-Durmont, *et al., Eur Jour Biochem*, (1996) 237:660-667). The cells are then treated so they differentiate into adipocytes as described above in Example I. An addition of an antibiotic selection marker allows enrichment for cells bearing the introduced genetic material. The derived adipocytes bearing the introduced genetic material may then be used to study gene regulation as described above.

The ability of the adipocyte to function as an endocrine organ has been validated, in part, by the discovery of leptin, a protein secreted almost exclusively by adipocytes which regulated energy homeostasis in mammals. Other examples include the ability of adipocytes to secrete vascular endothelial growth factor, the agouti protein, and angiotensinogen. All of these proteins/peptides have marked effects on wound healing

and angiogenesis, obesity, and cardiovascular function, respectively. The invention of a facile human adipocyte culture system allows the systematic identification of proteins and peptides secreted by these cells. Thus, in still another embodiment, the invention provides a method for identifying proteins and peptides secreted from cultured human adipocytes, comprising fractionating the conditioned media of adipocytes differentiated from preadipocytes by the methods of the invention.

In another aspect, the invention provides a method for preparing a three dimensional biomaterial containing adipose tissue-derived stromal cells capable of generating an adipose tissue depot upon implantation into a host recipient, comprising:

a) plating isolated human preadipocytes at a density of about 25,000 to 30,000 cells/cm^2 in a preadipocyte medium comprising a cell culture medium having or supplemented with 1.0-4.5 g/liter glucose and 0-15% fetal serum;

b) incubating said cells at about 37°C for about 4-48 hours until said cells are about 95-100% confluent;

c) replacing said preadipocyte medium with a differentiation medium comprising a cell culture medium having or supplemented with 1.0-4.5 g/liter glucose, 1-15% fetal serum; a cyclic AMP inducer; 100 nM to 1 μM insulin, or an equivalent amount of an insulin analogue; 16 nM to 1 μM of a glucocorticoid; and a concentration of a PPAR$_\gamma$ agonist or RXR agonist effective to stimulate differentiation of human preadipocytes;

d) incubating said cells at about 37°C for about 2-4 days;

e) introducing the cells from step (d) into an adipocyte medium containing a three-dimensional biocompatible matrix having interstitial spaces, wherein said adipocyte medium comprises a cell culture medium having or supplemented with 1.0-4.5 g/liter glucose; 0-15% fetal serum; 100 nM to 1 μM insulin, or an equivalent amount of an insulin analogue; 16 nM to 1 μM of a glucocorticoid; wherein said adipocyte medium contains neither an effective amount of a cAMP inducer nor a concentration of a PPAR$_\gamma$ agonist or RXR agonist effective to stimulate differentiation of human preadipocytes; and

f) incubating said cells at about 37°C for about 1-2 weeks and refeeding said cells with said adipocyte medium at least every 3-4 days;

wherein said preadipocyte medium, said differentiation medium and said adipocyte medium are maintained at a pH of about 7.0 to 7.6 when in contact with said cells.

The adipose biomatrices of the invention are particularly useful in the grafting of adipose tissue. Preferably the graft is autologous. Adipose tissue plays an important and overlooked role in the normal development and physiology of humans and other mammalian species. Many different kinds of fat exist. The most common type is white adipose tissue, located under the skin (subcutaneous fat), within the abdominal cavity (omental fat) and around the reproductive organs (gonadal fat). Less common in the adult human is brown adipose tissue, which plays an important role in generating heat during the neonatal period; this type of fat is located between the shoulder blades (interscapular), around the major vessels and heart (periaortic and pericardial), and above the kidney (suprarenal). As women mature, they develop increased amounts of mammary adipose tissue. The mammary fat pad serves as an energy source during periods of lactation. Indeed, reproductive capacity and maturation are closely linked to the adipose tissue stores of the individual. Puberty in women and men correlates closely with the production and release of leptin, an adipose tissue derived hormone, and to body fat composition. Other adipose tissue sites play a structural role in the body. For example, the mechanical fat pads in the soles of the feet provide a cushion against the impact of walking. Loss of this fat depot leads to progressive musculoskeletal damage and impaired mobility.

Obesity is currently the major disorder affecting people of all ages in the United States and other countries where calorie-rich diets and a sedentary lifestyle are common. Nevertheless, there are a significant number of individuals who are afflicted by conditions or diseases which result from an absence of adipose tissue. Lipodystrophy, either partial or generalized, is a potentially life-threatening illness and the most severe example. This disorder is most common in women and is characterized by a loss of subcutaneous adipose tissues. The results can be disfiguring. The nature of the disease remains poorly understood. In some families, there is a

strong genetic component with evidence of an autosomal trait [Peters JM, Barnes R, Bennett L, Gitomer WM, Bowcock AM, Garg A Nat Genet 18:292-295, 1998; Jackson SN, Pinkney J, Bargiotta A, Veal CD, Howlett TA, McNally PG, Corral R, Johnson A, Trembath RC Am J Hum Genet 63:534-540, 1998; Garg A, Wilson R, Barnes R, Arioglu E, Zaidi Z, Gurakan F, Kocak N, O'Rahilly S, Taylor SI, Patel SB, Bowcock AM J Clin Endocrinol Metab 84:3390-3394, 1999] Some cases may represent an autoimmune disease, since patients display abnormalities in their complement system.

Other cases appear to be the consequence of medication. Patients receiving HIV-1 protease inhibitors exhibit peripheral lipodystrophy [Carr A, Samaras K, Chisholm DJ, Cooper DA Lancet 351:1881-1883, 1998]. Lipodystrophy is not simply a cosmetic defect. Patients suffering from this disease also exhibit a complex metabolic dysregulation characterized by hyperlipidemia, hyperinsulinemia, and insulin resistant diabetes [Jackson et al Am J Hum Genet 63:534-540, 1998]. Because these patients respond poorly to insulin, their diabetes and its inherent complications cannot be controlled by conventional therapies. While the new oral anti-diabetic thiazolidinedione drugs offer a therapeutic option, additional treatments will be necessary. Recently described animal models of lipodystrophy may point the direction such treatments. Two different transgenic mice have been created where adipose tissue sites are greatly depleted [Shimomura I, Hammer RE, Richardson JA, Ikemoto S, Bashmakov Y, Goldstein JL, Brown MS Genes Dev 12:3182-3194, 1998; Moitra J, Mason MM, Olive M, Krylov D, Gavrilova O, Marcus-Samuels B, Feigenbaum L, Lee E, Aoyama T, Eckhaus M, Reitman ML, Vinson C 12:3168-3181, 1998]. In both sets of animals, this is accompanied by diabetes, >50-fold elevation in insulin levels, elevated serum triglycerides and free fatty acids. These animals demonstrate that the absence of fat can cause diabetes. Preliminary studies indicate that these mice can be successfully treated by implantation of exogenous adipose tissue from a histocompatible donor. This suggests that a similar treatment may be beneficial for patients suffering from a similar condition.

There are non-life threatening human conditions associated with an absence or loss of adipose tissue. While these disorders are cosmetic, they have a significant impact on quality of life issues in afflicted individuals. For example, facial acne in

adolescents can result in the loss of subcutaneous adipose tissue and severe disfiguring. Similar soft tissue scarring and defects can occur in patients receiving radiation as an adjunctive treatment for cancer. These can be treated by transplantation of the patient's own fat into the afflicted area [Billings E, May Plast Reconstruct Surg 83:368-381,1989;JW Bircoll M, Novack B Ann Plast Surg 18:327-329, 1987; Ellenbogen R Ann Plast Surg 16:179-194, 1986]. However, the success of these repair is often transient due to resorption of the transplanted cells by the surrounding tissue [Ersek RA Plast Reconstruct Surg 87:219-227,1991]. Some efforts to improve this have involved the incorporation of dextran beads adsorbed with basic fibroblast growth factor [Eppley BL, Sidner RA, Platis JM, Sadove AM Plast Reconstruct Surg 90:1022-1030, 1992]. Scars due to injury or surgery may have similar sequelae. The most extreme cases are those due to the treatment of life-threatening breast cancer, requiring either a lumpectomy or mastectomy. Some patients choose an artificial breast implant as a cosmetic option. Many prefer to undergo an extensive surgical transplantation of their abdominal adipose tissue and musculature to repair any disfigurement. These "TRAM flap" surgeries must be performed at the time of the mastectomy and cannot be repeated. Their success rate is variable. Impaired vascular supply to the transplanted tissue can lead to failure and resorption of the transplanted fat tissue.

Other uses for the three dimensional adipose matrices of the invention include, but are not limited to, screening the effectiveness and cytotoxicity of compounds, allergens, growth/regulatory factors, pharmaceutical compounds, etc., in vitro; elucidating the mechanism of certain diseases; studying the mechanism by which drugs and/or growth factors operate; diagnosing and monitoring cancer in a patient; gene therapy; and the production of biologically active products, to name but a few.

By " three dimensional biocompatible matrix" is meant any biocompatible compound, resorbable or non-resorbable, which is able support the adherence, growth, differentiation, proliferation, vascularization, and three-dimensional modeling of adipose tissue-derived preadipocyte stromal cells into a soft tissue or adipose tissue depot either in vivo or ex vivo. Preferred matrices include, but are not limited to, poly-lactic acid, poly-glycolic acid, alginate, matrigel, collagen type I and its derivatives, collagen type IV and its derivatives, any other collagen type and its

derivatives, and any combination thereof.

In one embodiment, the matrix is a poly lactic-co-glycolic disks having a porosity of 50 to 95%, more preferably the porosity is about 90%. Preferably, the thickness of the disk is 1 to 25 mm, more preferably the thickness is about 2.5 to 12.5 mm. Preferably the disk has a pore size of 50 to 1000 micrometers, more preferably the pore size is 200 to 600 micrometers.

Other matrix materials include, but are not limited to, nylon (polyamides), dacron (polyesters), polystyrene, polypropylene, polyacrylates, polyvinyl compounds (e.g., polyvinylchloride), polycarbonate (PVC), polytetrafluorethylene (PTFE, teflon), thermanox (TPX), nitrocellulose, cotton, polyglycolic acid (PGA), collagen (in the form of sponges, braids, or woven threads, etc.), cat gut sutures, cellulose, gelatin, or other naturally occurring biodegradable materials or synthetic materials, including, for example, a variety of biodegradable materials or synthetic materials, including, for example, a variety of polyhydroxyalkanoates. Any of these materials may be woven into a mesh, for example, to form the three-dimensional framework or scaffold. Certain materials, such as nylon, polystyrene, etc. are poor substrates for cellular attachment. When these materials are used as the three-dimensional framework, it is advisable to pre-treat the matrix prior to inoculation of stromal cells in order to enhance the attachment of stromal cells to the matrix. For example, prior to inoculation with stromal cells, nylon matrices could be treated with 0.1 M acetic acid and incubated in polylysine, PBS, and/or collagen to coat the nylon. Polystyrene could be similarly treated using sulfuric acid.

Where the cultures are to be maintained for long periods of time or cryopreserved, non-degradable materials such as nylon, dacron, polystyrene, polyacrylates, polyvinyls, teflons, cotton, etc., may be preferred. A convenient nylon mesh which could be used in accordance with the invention is Nitex, a nylon filtration mesh having an average pore size of 210 .mu.m and an average nylon fiber diameter of 90 .mu.m (#3-210/36 Tetko, Inc., N.Y.). Where the three-dimensional culture is itself to be implanted in vivo, it may be preferable to use biodegradable matrices such as polyglycolic acid, catgut suture material, collagen, or gelatin for example.

Where the cultured cells are to be used to transplantation or implantation in vivo, it is

preferable to obtain the stromal cells from the patient's own tissues. The growth of cells on the three-dimensional support may be further enhanced by adding to the framework or coating the framework with proteins (e.g., collagens, elastic fibers, reticular fibers) glycoproteins, glycosaminoglycans (e.g., heparin sulfate, chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan sulfate, keratin sulfate, etc.), a cellular matrix, and/or other materials.

Preferably, the cells are added to the matrix at a density of 100 to 100,000 cells per mm^3 of matrix as it exists in the culture conditions. The values are so described to account for the fact that a three-dimensional tissue is being developed. These values represent a range only and final concentrations must be determined by those skilled in the art to achieve maximal adipocyte differentiation in the implants.

The adipocyte medium can further comprise 10^{-9} to 10^{-3} molar of one or more compounds selected from the group consisting of: monobutyrin, glucocorticoids, and long chain fatty acids and/or 1 to 1000 ng/ml of one or more compounds selected from the group consisting of: vascular endothelial growth factor, fibroblast growth factor (beta), bone morphogenetic protein 4, bone morphogenetic protein 7, insulin, insulin-like growth factor I, insulin-like growth factor II, leptin and growth hormone.

The methods and composition have use in drug discovery for compounds and proteins with relevance to human conditions involving adipocytes. For example, the system developed can potentially be used to screen candidate drugs in an animal based model of human adipose tissue. With the development of successful human adipose tissue-derived stromal cell implants undergoing adipocyte differentiation in mice or rats, it will be possible to then screen their response to candidate drugs. This approach will allow the physiologic evaluation of drugs on adipocyte metabolism and will offer possible advantages over current methodologies examining the isolated human adipocyte in culture. Unlike cell culture systems, this method will permit interaction between adipocytes and other organ systems in terms of counter-regulatory mechanisms, positive and negative feedback loops, etc.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

Example 1

Differentiation of Human Preadipocytes into Adipocytes

Human preadipocytes were isolated from adipose tissue removed by liposuction surgery according to the procedures previously described by Rodbell and Hauner (Rodbell (1967) and (1974); Hauner, *supra*). Preadipocytes from the stroma-vascular fraction were resuspended in preadipocyte medium (DME-Ham's F-10, 1:1 (v/v), 10% FBS, and penicillin-streptomycin-fungizone) and plated at 25,000 cells/well in each of the wells of a 96 well plate (150 μl/well). The cells were then placed in a 37°C 5% CO_2 incubator and allowed to settle overnight. The following day, all of the medium was removed and replaced with 150 μl differentiation medium (Dulbecco's Modified Eagle Medium/Ham's F-10 Nutrient Broth (1:1, vol/vol), 15 mM HEPES buffer, pH 7.4, 33 μM biotin, 17 μM pantothenate, 0.2 mM isobutylmethylxanthine, 100 nM insulin, 1 μM dexamethasone, 1 μM BRL 49653 (diluted into medium from a 100X DMSO stock solution), 10% (vol/vol) fetal bovine serum, 60 U/l streptomycin, 60 U/l penicillin and 25 μg/l Fungizone). The cells were maintained on differentiation medium for 3 days. Additional differentiation medium was added as necessary to maintain the proper volume.

120 μl of the medium was removed, taking care to leave a 30 μl layer over the cell surface to prevent the maturing adipocytes from lifting off of the plate surface due to the surface tension created by removal of all the fluid. The cells were than fed with 120 μl adipocyte medium (Dulbecco's Modified Eagle/Ham's nutrient broth F-10 (1:1, v/v), 15 mM HEPES, pH 7.4, 3% fetal bovine serum, 33 μM biotin, 17 μM pantothenate, 100 nM insulin, 1 μM dexamethasone, 60 U/l penicillin, 60 U/l streptomycin and 25 μg/l Fungizone) and incubated at 37°C. The cells were refed with the adipocyte medium every 3-4 days for 14 days. At each feeding, 100 μl of old medium was removed and replaced with 100 μl fresh medium.

Oil droplets appeared approximately 4 days after differentiation medium was added. Adipocytes could be maintained for one week to three months by re-feeding with

adipocyte medium every three to four days. Figure 1 illustrates the normal and expected morphology observed for preadipocytes and adipocytes stained with oil red O.

The following procedure was used to determine the amount of lipid that had accumulated in the adipocytes. 120 μl/well of medium was removed from each well of the 96-well plates. Care was taken to prevent drying of the wells. 100 μl/ well fixer solution (7% formaldehyde in PBS) was added and the plate was incubated at room temperature for at lease 2 hours. Oil Red O working solution was prepared by adding 4 ml distilled water to 6 ml Oil Red O stock solution (1% Oil Red O in isopropanol). The working solution was incubated for 20 minutes at room temperature and then filtered through Nalgene 0.8 μm filter wear. All of the fixer was removed by aspiration until wells were completely dry.

The cells were then incubated with 40 μl/well Oil Red O working solution at room temperature for 10-15 minutes. Care was taken not to touch the sides of the wells in order to prevent high background due to dye stuck on the wall of the well. The plate was covered during incubation to prevent evaporation. Following this incubation, all of the Oil Red O solution was removed and the cells were washed four times with 200 μl/well distilled water. The wash water was drawn off from the top by inserting the manifold or pipette tips to the top of the liquid to remove Oil Red O precipitates floating at the surface. All of the remaining wash water was removed.

Isopropanol (100 μl/well) was then added and the plate was incubated at room temperature for 10 minutes. The isopropanol was pipetted up and down several times, to ensure that all of the Oil Red O was in solution. The optical density was measured at 500 nm. A five-fold increase in staining was observed for differentiated adipocytes as compared to preadipocytes (Figure 2). The average reading for stained adipocytes was 0.383 ± 0.020 OD_{500}. The average reading for the stained preadipocytes was 0.082 ± 0.014 OD_{500}. A uniform differentiation of cells in each plate was observed.

Example 2

Identification of Compounds that Enhance Preadipocyte Differentiation to

Adipocytes

To test the ability of a compound to stimulate differentiation of preadipocytes to adipocytes, the procedures of Example 1 were followed with the following modifications. BRL 49653 was omitted from the differentiation medium and replaced with the compound to be tested or with vehicle alone as a negative control. BRL 49653 was used as a positive control. Various concentrations of BRL49653 in DMEM/F-10 medium containing 3% fetal calf serum, 100 nM insulin, 1 μM dexamethasone, and 0.2 mM isobutylmethylxanthine were incubated with the cells for 3 days and then adipocyte media without BRL49653. After 9 days the cells were washed, fixed and stained with Oil Red O as described herein. The Oil Red O was extracted, quantitated spectrophotometrically and the results plotted as BRL49653 concentration versus optical density (OD). Figure 3 illustrates the dose responsive effect of BRL49653 on the ability to differentiate human preadipocytes as measured by Oil Red O staining.

Example 3

Identification of Glucocorticoids and Glucocorticoid Analogues

To test the ability of a compound to act as a glucocorticoid and/or glucocorticoid analogue, the procedures of Example 1 were followed with the following modifications. Human preadipocytes were isolated and cultured in DMEM/F-10 medium containing 3% fetal calf serum, 100 nM insulin, 0.2 mM isobutylmethylxanthine with increasing concentrations of dexamethasone under the conditions described herein for 3 days. The media was then replaced with DMEM/F10 (1:1) containing 3% fetal calf serum, 100 nM insulin and increasing concentrations of dexamethosome. The cells were fed with this medium every three days for 12 days. Cells were then washed, fixed and stained with Oil Red O as described above. Figure 4 illustrates the ability of dexamethasone to stimulate differentiation in a dose-dependent manner, as measured by lipid accumulation.

Example 4

Identification of PPAR$_\gamma$ Antagonists

To test the ability of compounds to act as a PPAR$_\gamma$ antagonist, the procedures of Example 1 were followed with the following modifications. The compound to be tested, or vehicle alone as a negative control, was added to the differentiation medium containing BRL49653 at 50 nM, which allows for half maximal stimulation of differentiation. In this case, human preadipocytes were isolated and cultured in DMEM/F-10 medium containing 3% fetal calf serum, 100 nM insulin, 50 nM BRL49653, 1 μM dexamethasone, 0.2 mM isobutylmethylxanthine and 1 μM of a number of novel compounds for 3 days. Cells were incubated with standard adipocyte medium for an additional 10 days. Cells were then washed, fixed and stained with Oil Red O as described.

Figure 5 illustrates the screening of a variety of novel compounds at a single dose to determine the ability to inhibit differentiation. The graph shows the optical density plotted versus the various compounds examined. The horizontal line at approximately 0.2 OD is the Oil Red O absorbance observed in the presence of 1 μM BRL49653, which is indicative of 100% differentiation. A number of compounds appear to inhibit half maximal differentiation as measured by Oil Red O staining, since 50 nM BRL4953 yields approximately 0.2 OD units (horizontal line).

Example 5

Identification of Compounds that Induce Lipolysis in Differentiated Adipocytes

Preadipocytes from 5 individual patients were isolated and differentiated into adipocytes as described herein. A 96-well plate of adipocytes differentiated by the method in Example 1 was prepared. After 21 days from plating, the cells were washed in Krebs-Ringer buffer and treated with increasing concentrations of isoproterenol. The positive and negative controls (0.5 μM isoproterenol in KRB and KRB alone,

respectively) were added in duplicate. The plates were then incubated at 37°C for four hours.

When lipolytic agents stimulate adipocytes, triglycerides are converted to glycerol and fatty acids. Lipolytic rate was measured in this example by measuring the glycerol released in the medium. At the end of the incubation time, conditioned medium (100μl) from each well was transferred into a new plate. Glycerol standards (1 mM, 0.5 mM, 0.25 mM, 0.1 mM, 0.05 mM, 0.025 mM, 0 mM) were included.

Glycerol assay reagent (100 μl; Sigma glycerol assay kit) was added to each well. The solutions were mixed well and incubated at room temperature for 15 minutes and then the optical density of each well was read at 540 nm. The increase in absorbance at 540 nm is directly proportional to glycerol concentration of the sample Figure 6 illustrates the ability of a known lipolytic agent, isoproterenol, to dose-dependently stimulate lipolysis in differentiated adipocytes isolated from five different patients.

Example 6

Introduction of DNA into Adipocytes

Adipocytes were differentiated from preadipocytes according to the method described in Example 1. 0.1-2.0 μg pCMV-βgal was added to 150 μl of Buffer EC (Qiagen Effectene kit, Catalog number 301425, Qiagen, Valencia CA) and then the DNA was allowed to condense in a microcentrifuge tube. 8 μl enhancer (Qiagen Effectene kit) was then added to the DNA mixture, vortexed for one second and allowed to incubate at room temperature for 2-5 minutes. This mixture was centrifuged briefly to remove drops at the top of the tube. 10 μl Effectene reagent was then added, vortexed for 10 seconds, and incubated at room temperature for 5-10 minutes. 120 μl of the old medium was removed (30 μl should be left in the well) from the cells. 70 μl of fresh adipocyte medium was then added. Following a 5-10 minute incubation time, 1 ml of medium was added to the DNA mixture. 25 μl of this final mixture was added to each well. The cells were then incubated for 5 hours. However, Effectene is not toxic and may be left on the

cells for any period of time. The cells were then rinsed cells with 80 μl adipocyte medium and then assayed at 72 hours post-infection for β-galactosidase activity. Figure 7 illustrates the dose-dependence of DNA concentration and the effect of time in culture on the transfection efficiency using Effectene, as measured by β-galactosidase activity.

Example 7

Identification of Novel Proteins from Human Cultured Adipocytes and Preadipocytes

The following protocol allows identification of novel proteins from adipocytes prepared according to the methods described in Example 1. Preadipocytes and differentiated adipocytes from the same patient were cultured as described in Example 1 and then incubated in methionine-free adipocyte media for 2 hours. 100 μCi ^{35}[S] methionine was then added to the media and cells were incubated with this mixture for 2 hrs. This media was then replaced with adipocyte media containing a 1 mM non-radioactive methionine chase.

To determine the pattern of secreted proteins, 10 ml of conditioned media was removed from the cells and dialyzed against 4 liters of cold KRB at 4°C for 4 hrs to remove unincorporated ^{35}[S]methionine from the radiolabeled proteins. Protein concentration and total radioactivity were determined and 50,000 cpm from each sample was mixed 1:1 vol/vol with a 2X dilution buffer (16% NP-40, 2% ampholines, 8 M urea, 7.5 % β-mercaptoethanol, 0.1 % bromophenol blue) and immediately loaded onto 12.5 cm long 3% acrylamide gel tube gels containing 8 M urea, 10% NP-40, 5.5% ampholines. Three to four crystals of urea were added to the samples and the tubes agitated until dissolved.

The tubes were placed in an isoelectric focusing chamber with the bottom chamber containing 0.01M H_3P0_4 and the top chamber containing 0.02 M NaOH. The samples were run at 12,000 V-hours at constant amperage (0.25 mA/tube). When the voltage increased to 500 volts, the power control was changed to constant voltage. After 12-16 hrs, the voltage was increased to 1000 volts for 3 hours to focus the proteins.

The tube gels were extruded from their cylinders and incubated in equilibration buffer (10% glycerol, 5% β-mercaptoethanol, 0.1 M Tris, pH 6.8, 2% sodium dodecyl sulfate) for 20 minutes. The gradient gel was then placed horizontally onto a 7.5 % polyacrylamide gel containing 0.1 M Tris-HCl pH 6.8 and 1% SDS for separation via apparent molecular weight.

The gel was run for 4 hours at 120 volts, removed from the plates and fixed in acetic acid:methanol:water (1:7:2 vol/vol) overnight. The gel was then soaked in a 10% glycerol solution for 20 minutes and dried under vacuum and heat. The dried gel was exposed to x-ray film for 24 hrs to determine the pattern of radiolabeled protein on the gel. Figure 8 illustrates the patterns observed after labeling secreted proteins from both human preadipocytes (2 days) and differentiated adipocytes (14 days) from the same patient.

Example 8

Generation of Three Dimensional Adipose Tissue *ex vivo*.

Stromal cells are isolated from human subcutaneous adipose tissue according to methods described above. Briefly, the cells are plated at a density of 500 to 20,000 cells per cm^2. One day after plating, the cells are converted to "Adipocyte Differentiation Medium" containing insulin, biotin, pantothenate, BRL49,653, dexamethasone, and isobutylmethylxanthine or equivalent compounds at concentrations known to those skilled in the art. After an additional three days in culture in this medium, the cells are harvested by trypsin/EDTA digestion at 37° C for a period of 1 to 15 minutes, and suspended in an equal volume of medium containing a minimum of 10% fetal bovine serum. The cells are centrifuged at 100 to 500 X g, preferably at 282 X g for 1 to 10 minutes, preferably for 5 minutes at 4° C to 37° C, preferably at 20° C. The concentrated cells are resuspended in Adipocyte Maintainence Medium containing 1000 to 10,000 mg glucose per liter, preferably 4500 mg/liter, 1 to 10% fetal bovine serum, preferably 3% fetal bovine serum, insulin, biotin, pantothenate, and dexamethasone at concentrations known to those skilled in the art. The cells are

resuspended at a concentration between 1000 to 10 million cells per ml, more preferably at 100,000 to 1 million per ml, most preferably at 250,000 cells per ml.

The concentrated cells are pipetted directly onto a poly lactic-co-glycolic polymer disk of porosity 50% to 95%, preferably at 90%, with a thickness between 1 to 10 mm, most preferably 2.5 mm, and a diameter of 5 to 25 mm, most preferably 12.5 mm, with a pore size range of 50 to 1000 mum, most preferably 200 to 600 mum. Matrices can be synthesized with the biomaterial alone or with incorporated adipocyte inducing materials. These include, but are not limited to, adipogenic compounds (such as the thiazolidinedione BRL49,653, growth hormone, dexamethasone, basic fibroblast growth factor, and/or MAP Kinase inhibitors), vascularization compounds (such as monobutyrin), and/or expression vectors containing cDNAs encoding adipogenic factors (such as constitutively active peroxisome proliferator activated receptor gamma2 or constitutively active bone morphogenetic protein receptor IA) or vascularization inducing factors (such as vascular endothelial growth factor). Alternatively, cells are resuspended in Matrigel or in another alternative biocompatible material such as alginate at concentrations between 100,000 to 1 million per ml; these may also incorporate additional factors as described above. The cells in the 3-dimensional matrix are maintained in Adipocyte Maintainence Medium continuously and replaced with fresh medium every 2 to 4 days, most preferably every 3rd day. Cell differentiation along the adipocyte lineage is monitored by the appearance of lipid vacuoles based on phase contrast microscopy and by staining with oil red O, according to "Methods and Composition for the Differentiation of Human Preadipocytes into Adipocytes" Serial Number 09/240,029 filed January 29, 1999. The 3-dimensional matrices can be examined directly by light microscopy or embedded in paraffin for sectioning. Additional methods to monitor adipogenesis include detection of adipocyte specific gene markers such as, but not limited to, adipocyte fatty acid binding protein aP2, leptin, lipoprotein lipase, and adipsin by northern blot, western blot, ELISA, and PCR analyses. These methods can be used to optimize conditions for adipocyte differentiation *ex vivo* and to determine the length of time permitted for maximal adipogenic commitment prior to implantation or *in vivo* studies.

Example 9

Generation of Implantable Three-Dimensional Adipose Tissue Depots *in vivo* Using Immunodeficient Rodent Models

The three-dimensional stromal cell matrices developed in Example 8 above are employed for *in vivo* implantation. Immunodeficient rodent models include, but are not limited to, severe combined immunodeficient (SCID) mice, nude mice, nude/beige mice, SCID/non-obese diabetic (NOD) mice, and nude rats. Two methods are described below but these are not exclusive of alternative approaches. In the first method, harvested stromal cells are maintained in culture for one to three passages to obtain maximal numbers of cells. Stromal cells are isolated from human subcutaneous adipose tissue according to methods described in "Methods and Composition for the Differentiation of Human Preadipocytes into Adipocytes" Serial Number 09/240,029 filed January 29, 1999. The cells are plated at a density of 500 to 20,000 cells per cm^2. The undifferentiated preadipocyte stromal cells are are harvested by trypsin/EDTA digestion at 37° C for a period of 1 to 15 minutes, and suspended in an equal volume of medium containing a minimum of 10% fetal bovine serum. The cells are centrifuged at 100 to 500 X g, preferably at 282 X g for 1 to 10 minutes, preferably for 5 minutes at 4° C to 37° C, preferably at 20° C. The concentrated cells are resuspended in Adipocyte Maintainence Medium containing 1000 to 10,000 mg glucose per liter, preferably 4500 mg/liter, 1 to 10% fetal bovine serum, preferably 3% fetal bovine serum, insulin, biotin, pantothenate, and dexamethasone at concentrations known to those skilled in the art. The cells are resuspended at a concentration between 1000 to 10 million cells per ml, more preferably at 100,000 to 1 million per ml, most preferably at 250,000 cells per ml. The concentrated cells are pipetted directly onto a poly lactic-co-glycolic polymer disk of porosity 50% to 95%, preferably at 90%, with a thickness between 1 to 10 mm, most preferably 2.5 mm, and a diameter of 5 to 25 mm, most preferably 12.5 mm, with a pore size range of 50 to 1000 mum, most preferably 200 to 600 mum. Matrices can be synthesized with the biomaterial alone or with incorporated adipocyte inducing materials. These include,

but are not limited to, adipogenic compounds (such as the thiazolidinedione BRL49,653, growth hormone, dexamethasone, basic fibroblast growth factor, and/or MAP Kinase inhibitors), vascularization compounds (such as monobutyrin), and/or expression vectors containing cDNAs encoding adipogenic factors (such as constitutively active peroxisome proliferator activated receptor gamma2 or constitutively active bone morphogenetic protein receptor IA) or vascularization inducing factors (such as vascular endothelial growth factor). Alternatively, cells are resuspended in Matrigel or in another alternative biocompatible material such as alginate at concentrations between 100,000 to 1 million per ml; these may also incorporate additional factors as described above.

In the second method, the cells are permitted to initiate adipocyte differentiation prior to incorporation into the three dimensional matrix. Stromal cells are isolated from human subcutaneous adipose tissue according to methods described in "Methods and Composition for the Differentiation of Human Preadipocytes into Adipocytes" Serial Number 09/240,029 filed January 29, 1999. The cells are plated at a density of 500 to 20,000 cells per cm^2.One day after plating, the cells are converted to "Adipocyte Differentiation Medium" containing insulin, biotin, pantothenate, BRL49,653, dexamethasone, and isobutylmethylxanthine or equivalent compounds at concentrations known to those skilled in the art. After an additional three days in culture in this medium, the cells are harvested by trypsin/EDTA digestion at 37° C for a period of 1 to 15 minutes, and suspended in an equal volume of medium containing a minimum of 10% fetal bovine serum. The cells are centrifuged at 100 to 500 X g, preferably at 282 X g for 1 to 10 minutes, preferably for 5 minutes at 4° C to 37° C, preferably at 20° C. The concentrated cells are resuspended in Adipocyte Maintainence Medium containing 1000 to 10,000 mg glucose per liter, preferably 4500 mg/liter, 1 to 10% fetal bovine serum, preferably 3% fetal bovine serum, insulin, biotin, pantothenate, and dexamethasone at concentrations known to those skilled in the art. The cells are resuspended at a concentration between 1000 to 10 million cells per ml, more preferably at 100,000 to 1 million per ml, most preferably at 250,000 cells per ml. The concentrated cells are pipetted directly onto a poly lactic-co-glycolic polymer disk of porosity 50% to 95%, preferably at 90%, with a thickness between 1 to 10

mm, most preferably 2.5 mm, and a diameter of 5 to 25 mm, most preferably 12.5 mm, with a pore size range of 50 to 1000 mum, most preferably 200 to 600 mum. Matrices can be synthesized with the biomaterial alone or with incorporated adipocyte inducing materials. These include, but are not limited to, adipogenic compounds (such as the thiazolidinedione BRL49,653, growth hormone, dexamethasone, basic fibroblast growth factor, and/or MAP Kinase inhibitors), vascularization compounds (such as monobutyrin), and/or expression vectors containing cDNAs encoding adipogenic factors (such as constitutively active peroxisome proliferator activated receptor gamma2 or constitutively active bone morphogenetic protein receptor IA) or vascularization inducing factors (such as vascular endothelial growth factor). Alternatively, cells are resuspended in Matrigel or in another alternative biocompatible material such as alginate at concentrations between 100,000 to 1 million per ml; these may also incorporate additional factors as described above.

Prior to implantation, cells can be marked by exposure to adenoviral vectors expressing the green fluorescent protein, beta-galactosidase, or other marker protein or enzyme, by exposure to retroviral vectors expressing the same markers, by exposure fluorescent probes, or by other standard or newly developed methodologies. These methods permit the identification of the donor cells in the host recipient animal at later time.

The resulting three dimensional matrices are implanted subcutaneously in one of the immunodeficient rodent models described above. Animals are anesthetized with ketamine and rompine or an equivalent anesthetic/analgesic by intraperitoneal injection or a veterinarian approved and reviewed alternative administration method. Studies are conducted with the review and approval of an insitutitional animal care and utilization committee. Implants are maintained for periods of 1 day to 12 months, more preferably 3 weeks to 12 weeks, most preferably for 5 weeks. Animals are fed a regular chow diet (4-5% fat), a high fat diet (10-30% fat, either omega-3 or omega-6 enriched), a high carbohydrate diet (>50% carbohydrate), or a high fat/high carbohydrate diet during part or all of this period. The presence of human adipocytes in the animals is detected during this period by collection of serum and ELISA assay for the human form of the adipocyte specific hormone, leptin. At the conclusion of the

study, implants are harvested by surgical removal and analyzed by histochemical, immunofluorescent, biochemical, and molecular biological techniques for the appearance of adipocytes or fat cells in the implant site. The presence of differentiated human adipocytes is determined by detection of the unique human DNA gene marker, the "alu" fragment, using in situ PCR methods.

In addition, methods to detect any marker proteins, enzymes or fluorescent probes are utilized to document the presence of donor cells in the final differentiated implant. The cellular composition, size, and viability of the implant is determined at this time. These methods are used to optimize the growth conditions, factors, proteins, cDNAs, and biomaterials necessary to support adipocyte differentiation by the donor human stromal cells in the host animal.

This approach can be modified to prepare a selective modeled three dimensional implant. The biomaterial can be shaped to meet specifications required for a particular need. To test this approach, biocompatible polymers are prepared with varying widths, heights and thicknesses to determine the ability to create "designer" soft tissue depots. The degree of these tests in rodents may be limited. Alternative large animal models (dogs, pigs, sheep) will be considered to test the dimensional limits facing this tissue engineering approach. The volume ratio of the tissue depot may correlate with the actual size of the host animal and may not reflect the geometry of the implant itself.

Example 10

Generation of Injectable Three-Dimensional Adipose Tissue Depots *in vivo* Using Immunodeficient Rodent Models

The three-dimensional stromal cell matrices developed in Example 8 above are employed for *in vivo* implantation. Immunodeficient rodent models include, but are not limited to, severe combined immunodeficient (SCID) mice, nude mice, nude/beige mice, SCID/non-obese diabetic (NOD) mice, and nude rats. Two methods are described below but these are not exclusive of alternative approaches. In the first method, harvested stromal cells are maintained in culture for one to three passages to obtain maximal numbers of cells. Stromal cells are isolated from human subcutaneous

adipose tissue according to methods described in "Methods and Composition for the Differentiation of Human Preadipocytes into Adipocytes" Serial Number 09/240,029 filed January 29, 1999. The cells are plated at a density of 500 to 20,000 cells per cm^2. The undifferentiated preadipocyte stromal cells are are harvested by trypsin/EDTA digestion at 37° C for a period of 1 to 15 minutes, and suspended in an equal volume of medium containing a minimum of 10% fetal bovine serum. The cells are centrifuged at 100 to 500 X g, preferably at 282 X g for 1 to 10 minutes, preferably for 5 minutes at 4° C to 37° C, preferably at 20° C. The concentrated cells are resuspended in Adipocyte Maintainence Medium containing 1000 to 10,000 mg glucose per liter, preferably 4500 mg/liter, 1 to 10% fetal bovine serum, preferably 3% fetal bovine serum, insulin, biotin, pantothenate, and dexamethasone at concentrations known to those skilled in the art. The cells are resuspended at a concentration between 1000 to 10 million cells per ml, more preferably at 100,000 to 1 million per ml, most preferably at 2 million cells per ml.

In the second method, the cells are permitted to initiate adipocyte differentiation prior to incorporation into the three dimensional matrix. Stromal cells are isolated from human subcutaneous adipose tissue according to methods described in "Methods and Composition for the Differentiation of Human Preadipocytes into Adipocytes" Serial Number 09/240,029 filed January 29, 1999. The cells are plated at a density of 500 to 20,000 cells per cm^2.One day after plating, the cells are converted to "Adipocyte Differentiation Medium" containing insulin, biotin, pantothenate, BRL49,653, dexamethasone, and isobutylmethylxanthine or equivalent compounds at concentrations known to those skilled in the art. After an additional three days in culture in this medium, the cells are harvested by trypsin/EDTA digestion at 37° C for a period of 1 to 15 minutes, and suspended in an equal volume of medium containing a minimum of 10% fetal bovine serum. The cells are centrifuged at 100 to 500 X g, preferably at 282 X g for 1 to 10 minutes, preferably for 5 minutes at 4° C to 37° C, preferably at 20° C. The concentrated cells are resuspended in Adipocyte Maintainence Medium containing 1000 to 10,000 mg glucose per liter, preferably 4500 mg/liter, 1 to 10% fetal bovine serum, preferably 3% fetal bovine serum, insulin, biotin, pantothenate, and dexamethasone at concentrations known to those skilled in the art.

The cells are resuspended at a concentration between 1000 to 10 million cells per ml, more preferably at 100,000 to 1 million per ml, most preferably at 2 million cells per ml.

Prior to implantation, cells can be marked by exposure to adenoviral vectors expressing the green fluorescent protein, beta-galactosidase, or other marker protein or enzyme, by exposure to retroviral vectors expressing the same markers, by exposure fluorescent probes, or by other standard or newly developed methodologies. These methods permit the identification of the donor cells in the host recipient animal at later time.

The resulting cells from either method are mixed with liquid Matrigel (collagen type IV) or other liquid biocompatible polymer at a cell concentration of between 50,000 to 5 million cells per ml, most preferably at one million cells per ml, and a Matrigel concentration of 5 to 20 mg per ml, most preferably at 10 mg per ml. The suspensions can be synthesized with the biomaterial alone or with incorporated adipocyte inducing materials. These include, but are not limited to, adipogenic compounds (such as the thiazolidinedione BRL49,653, growth hormone, dexamethasone, basic fibroblast growth factor, and/or MAP Kinase inhibitors), vascularization compounds (such as monobutyrin), and/or expression vectors containing cDNAs encoding adipogenic factors (such as constitutively active peroxisome proliferator activated receptor gamma2 or constitutively active bone morphogenetic protein receptor IA) or vascularization inducing factors (such as vascular endothelial growth factor).

The cell/Matrigel suspension is then injected subcutaneously into one of the immunodeficient rodent models described above. Prior to injection, animals are anesthetized with ketamine and rompine or an equivalent anesthetic/analgesic by intraperitoneal injection or a veterinarian approved and reviewed alternative administration method. Studies are conducted with the review and approval of an insitutitional animal care and utilization committee. Implants are maintained for periods of 1 day to 12 months, more preferably 3 weeks to 12 weeks, most preferably for 5 weeks. Animals are fed a regular chow diet (4-5% fat), a high fat diet (10-30% fat, either omega-3 or omega-6 enriched), a high carbohydrate diet (>50%

carbohydrate), or a high fat/high carbohydrate diet during part or all of this period. The presence of human adipocytes in the animals is detected during this period by collection of serum and ELISA assay for the human form of the adipocyte specific hormone, leptin. At the conclusion of the study, implants are harvested by surgical removal and analyzed by histochemical, immunofluorescent, biochemical, and molecular biological techniques for the appearance of adipocytes or fat cells in the implant site. The presence of differentiated human adipocytes is determined by detection of the unique human DNA gene marker, the "alu" fragment, using in situ PCR methods.

In addition, methods to detect any marker proteins, enzymes or fluorescent probes are utilized to document the presence of donor cells in the final differentiated implant. The cellular composition, size, and viability of the implant is determined at this time. These methods are used to optimize the growth conditions, factors, proteins, cDNAs, and biomaterials necessary to support adipocyte differentiation by the donor human stromal cells in the host animal.

Example 11

Utilization of Three-Dimensional Human Adipose Tissue Depots in Animal Models for Screening and Testing of Therapeutic Compounds.

Previous studies of human adipose tissue are limited to two dimensional cultures containing isolated human adipocytes *in vitro* unless the studies have progressed to a Phase I clinical trial. These methods are described above. The animal models described in Examples 2 and 3 above can be used to screen and/or test novel or existing therapeutic compounds to determine their effect on human adipose tissue under physiologic conditions. The immunodeficient rodent models provide a "humanized" adipose tissue depot for analysis in the context of physiologic conditions. The following description is only an example and should not be construed as limiting or exclusive in any way. To test the effect of a novel thiazolidinedione or peroxisome proliferator activated receptor gamma 2 agonist on adipocyte function in a diabetic, it is possible to create a diabetic model using rodents with a "humanized adipose depot"

model. The rodents can be treated with streptozocin to achieve a chemically induced pancreatectomy. Animals can be monitored for the development of diabetes based on serum glucose levels. Once diabetes is established, the animals can be divided into a control group, receiving no treatment, and experimental grouops, receiving the test compound at varying doses and by varying routes of administration. The effect of the test compound on the animals diabetes can be monitored non-invasively by serum glucose determinations. This can be correlated with the serum levels of human leptin based on ELISA assays exclusively detecting human leptin protein and distinguishing it from any rodent leptin protein as well as with serum apolipoprotein levels (human and rodent), triglycerides, free fatty acids, and ketone levels. At the conclusion of the study, the size, composition, and metabolic activity of the humanized adipose tissue depot can be compared between the experimental and control animal groups. This approach allows the testing of potential therapeutic compounds on human adipose tissue prior to FDA approval of any Phase I studies. It has the potential of allowing pharmaceutical companies to optimize their opportunity for success at this important juncture in the drug discovery process.

Many modifications and other embodiments of the invention will come to mind to one skilled in the art to which this invention pertains having the benefit of the teachings presented in the foregoing descriptions and the associated drawings. Therefore, it is to be understood that the invention is not to be limited to the specific embodiments disclosed and that modifications and other embodiments are intended to be included within the scope of the appended claims. Although specific terms are employed herein, they are used in a generic and descriptive sense only and not for purposes of limitation.

THAT WHICH IS CLAIMED:

1. A method for differentiating human preadipocytes into adipocytes, comprising:

a) plating isolated human preadipocytes at a density of about 25,000 to 30,000 cells/cm^2 in a preadipocyte medium comprising a cell culture medium having or supplemented with 1.0-4.5 g/liter glucose and 0-15% fetal serum;

b) incubating said cells at about 37°C for about 4-48 hours until said cells are about 95-100% confluent;

c) replacing said preadipocyte medium with a differentiation medium comprising a cell culture medium having or supplemented with 1.0-4.5 g/liter glucose, 1-15% fetal serum; a cyclic AMP inducer; 100 nM to 1 μM insulin, or an equivalent amount of an insulin analogue; 16 nM to 1 μM of a glucocorticoid; and a concentration of a PPAR$_\gamma$ agonist or RXR agonist effective to stimulate differentiation of human preadipocytes;

d) incubating said cells at about 37°C for about 2-4 days;

e) replacing said differentiation medium with an adipocyte medium comprising a cell culture medium having or supplemented with 1.0-4.5 g/liter glucose; 0-15% fetal serum; 100 nM to 1 μM insulin, or an equivalent amount of an insulin analogue; 16 nM to 1 μM of a glucocorticoid; wherein said adipocyte medium contains neither an effective amount of a cAMP inducer nor a concentration of a PPAR$_\gamma$ agonist or RXR agonist effective to stimulate differentiation of human preadipocytes; and

f) incubating said cells at about 37°C for about 1-2 weeks and refeeding said cells with said adipocyte medium at least every 3-4 days;

wherein said preadipocyte medium, said differentiation medium and said adipocyte medium are maintained at a pH of about 7.0 to 7.6 when in contact with said cells.

2. The method of claim 1, wherein said cAMP inducer is 0.2 to 0.5 mM isobutylmethylxanthine.

3. The method of claim 1, wherein said preadipocyte medium, said differentiation medium and said adipocyte medium comprise 3-10% FBS.

4. The method of claim 1, wherein said differentiation medium and said adipocyte medium further comprise 1-100 μM pantothenate and 1-100 μM biotin.

5. The method of claim 1, wherein said cell culture medium is DME/Ham's F-10 Nutrient Broth (1:1 vol/vol).

6. The method of claim 1, wherein said $PPAR_\gamma$ agonist is a thiazolidinedione.

7. The method of claim 6, wherein said thiazolidinedione is BRL 49653.

8. The method of claim 7, wherein the concentration of said BRL 49653 is 0.5-1.0 μM.

9. The method of claim 6 wherein said thiazolidinedione is troglitazone.

10. The method of claim 9, wherein the concentration of said troglitazone is 1-5 μM.

11. The method of claim 1, wherein said glucocorticoid is dexamethasone, hydrocortisone or cortisol.

12. The method of claim 1, wherein said cyclic AMP inducer is isobutylmethylxanthine or forskolin.

13. A method for determining the ability of a compound to affect the differentiation of preadipocytes to adipocytes, comprising:

a) plating isolated human preadipocytes at a density of about 25,000 to 30,000 cells/cm^2 in a preadipocyte medium comprising a cell culture medium having or supplemented with 1.0-4.5 g/liter glucose and 0-15% fetal serum;

b) incubating said cells at about 37°C for about 4-48 hours until said cells are about 95-100% confluent;

c) replacing said preadipocyte medium with a differentiation medium comprising a cell culture medium having or supplemented with 1.0-4.5 g/liter glucose, 1-15% fetal serum; a cyclic AMP inducer; 100 nM to 1 μM insulin, or an equivalent amount of an insulin analogue; 16 nM to 1 μM of a glucocorticoid; a concentration of a PPAR$_\gamma$ agonist or RXR agonist effective to stimulate differentiation of human preadipocytes; and said compound in an appropriate vehicle or vehicle alone;

d) incubating said cells at about 37°C for about 2-4 days;

e) replacing said differentiation medium with an adipocyte medium comprising a cell culture medium having or supplemented with 1.0-4.5 g/liter glucose; 0-15% fetal serum; 100 nM to 1 μM insulin, or an equivalent amount of an insulin analogue; 16 nM to 1 μM of a glucocorticoid; wherein said adipocyte medium contains neither an effective amount of a cAMP inducer nor a concentration of a PPAR$_\gamma$ agonist or RXR agonist effective to stimulate differentiation of human preadipocytes; and

f) incubating said cells at about 37°C for about 1-2 weeks and refeeding said cells with said adipocyte medium at least every 3-4 days; and

g) determining the number or percentage of differentiated cells in the cells from step (f) fed at step (c) with said differentiation medium containing said compound;

h) determining the number or percentage of differentiated cells in the cells from step (f) fed at step (c) with said differentiation medium containing said vehicle alone; and

i) comparing the number or percentage of differentiated cells from steps (g) and (h);

wherein said preadipocyte medium, said differentiation medium and said adipocyte medium are maintained at a pH of about 7.0 to 7.6 when in contact with said cells.

14. A method for determining the ability of a compound to act as a $PPAR_\gamma$ antagonist, comprising:

a) plating isolated human preadipocytes at a density of about 25,000 to 30,000 cells/cm^2 in a preadipocyte medium comprising a cell culture medium having or supplemented with 1.0-4.5 g/liter glucose and 0-15% fetal serum;

b) incubating said cells at about 37°C for about 4-48 hours until said cells are about 95-100% confluent;

c) replacing said preadipocyte medium with a differentiation medium comprising a cell culture medium having or supplemented with 1.0-4.5 g/liter glucose, 1-15% fetal serum; a cyclic AMP inducer; 100 nM to 1 μM insulin, or an equivalent amount of an insulin analogue; 16 nM to 1 μM of a glucocorticoid;and a concentration of a $PPAR_\gamma$ agonist effective to stimulate half-maximal differentiation of a human preadipocytes; and said compound in an appropriate vehicle or vehicle alone;

d) Incubating said cells at about 37°C for about 2-4 days;

e) replacing said differentiation medium with an adipocyte medium comprising a cell culture medium having or supplemented with 1.0-4.5 g/liter glucose; 0-15% fetal serum; 100 nM to 1 μM insulin, or an equivalent amount of an insulin analogue; 16 nM to 1 μM of a glucocorticoid; wherein said adipocyte medium contains neither an effective amount of a cAMP inducer nor a concentration of a $PPAR_\gamma$ agonist or RXR agonist effective to stimulate differentiation of human preadipocytes; and

f) Incubating said cells at about 37°C for about one week and refeeding said cells at least once with the supplemented medium from step (e); and

g) Determining the number or percentage of differentiated cells in the cells from step (f) fed at step (c) with medium containing said compound;

h) Determining the number or percentage of differentiated cells in the cells from step (f) fed at step (c) with medium containing said vehicle alone; and

i) Comparing the number or percentage of differentiated cells from steps (g) and (h);

wherein said preadipocyte medium, said differentiation medium and said adipocyte medium are maintained at a pH of about 7.0 to 7.6 when in contact with said cells.

15. A method for determining the ability of a compound to act as an insulin analogue, comprising:

a) plating isolated human preadipocytes at a density of about 25,000 to 30,000 cells/cm^2 in a preadipocyte medium comprising a cell culture medium having or supplemented with 1.0-4.5 g/liter glucose and 0-15% fetal serum;

b) incubating said cells at about 37°C for about 4-48 hours until said cells are about 95-100% confluent;

c) replacing said preadipocyte medium with a differentiation medium comprising a cell culture medium having or supplemented with 1.0-4.5 g/liter glucose, 1-15% fetal serum; a cyclic AMP inducer; 16 nM to 1 μM of a glucocorticoid; and a concentration of a $PPAR_\gamma$ agonist or RXR agonist effective to stimulate differentiation of a human preadipocytes; and said compound in an appropriate vehicle or vehicle alone;

d) incubating said cells at about 37°C for about 2-4 days;

e) replacing said differentiation medium with an adipocyte medium comprising a cell culture medium having or supplemented with 1.0-4.5 g/liter glucose; 0-15% fetal serum; 16 nM to 1 μM of a glucocorticoid; and said compound in an appropriate vehicle or vehicle alone; wherein said adipocyte medium contains neither an effective amount of a cAMP inducer nor a concentration of a $PPAR_\gamma$ agonist or RXR agonist effective to stimulate differentiation of human preadipocytes;

f) incubating said cells at about 37°C for about 1-2 weeks and refeeding said cells at least every 3-4 days with said adipocyte medium; and

g) determining the number or percentage of differentiated cells in the cells from step (f) fed at step (c) with medium containing said compound;

h) determining the number or percentage of differentiated cells in the cells from step (f) fed at step (c) with medium containing said vehicle alone; and

i) comparing the number or percentage of differentiated cells from steps (g) and (h);

wherein said preadipocyte medium, said differentiation medium and said adipocyte medium are maintained at a pH of about 7.0 to 7.6 when in contact with said cells.

16. A cultured human adipocyte prepared by the method of claim 1.

17. The adipocyte of claim 16, wherein said adipocyte is transiently or stably transfected with at least one nucleic acid sequence.

18. A method for identifying polypeptides secreted from cultured human adipocytes, comprising: fractionating the polypeptides secreted by the adipocyte of claim 16.

19. A method for preparing a three dimensional biomaterial containing adipose tissue-derived stromal cells capable of generating an adipose tissue depot upon implantation into a host recipient, comprising:

a) plating isolated human preadipocytes at a density of about 25,000 to 30,000 cells/cm^2 in a preadipocyte medium comprising a cell culture medium having or supplemented with 1.0-4.5 g/liter glucose and 0-15% fetal serum;

b) incubating said cells at about 37°C for about 4-48 hours until said cells are about 95-100% confluent;

c) replacing said preadipocyte medium with a differentiation medium comprising a cell culture medium having or supplemented with 1.0-4.5 g/liter glucose, 1-15% fetal serum; a cyclic AMP inducer; 100 nM to 1 μM insulin, or an equivalent amount of an insulin analogue; 16 nM to 1 μM of a glucocorticoid; and a concentration of a $PPAR_\gamma$ agonist or RXR agonist effective to stimulate differentiation of human preadipocytes;

d) incubating said cells at about 37°C for about 2-4 days;

e) introducing the cells from step (d) into an adipocyte medium containing a three-dimensional biocompatible matrix having interstitial spaces, wherein said adipocyte medium comprises a cell culture medium having or supplemented with 1.0-4.5 g/liter glucose; 0-15% fetal serum; 100 nM to 1 μM insulin, or an equivalent amount of an insulin analogue; 16 nM to 1 μM of a glucocorticoid; wherein said adipocyte medium contains neither an effective amount of a cAMP inducer nor a concentration of a $PPAR_\gamma$ agonist or RXR agonist effective to stimulate differentiation of human preadipocytes; and

f) incubating said cells at about 37°C for about 1-2 weeks and refeeding said cells with said adipocyte medium at least every 3-4 days;

wherein said preadipocyte medium, said differentiation medium and said adipocyte medium are maintained at a pH of about 7.0 to 7.6 when in contact with said cells.

20. The method of claim 19, wherein said matrix comprises poly-lactic acid, poly-glycolic acid or collagen, matrigel, or a combination thereof.

21. The method of claim 19, wherein said adipocyte medium further comprises 10^{-9} to 10^{-3} molar of one or more compounds selected from the group consisting of: monobutyrin, glucocorticoids, and long chain fatty acids.

22. The method of claim 19, wherein said adipocyte medium further comprises 1 to 1000 ng/ml of one or more compounds selected from the group consisting of: vascular endothelial growth factor, fibroblast growth factor (beta), bone morphogenetic protein 4, bone morphogenetic protein 7, insulin, insulin-like growth factor I, insulin-like growth factor II, leptin and growth hormone.

23. The method of claim 19, wherein the said cells are introduced into said adipocyte medium at a density of 100 to 100,000 cells per mm^3 of said matrix.

24. The method of claim 19, wherein said adipocyte medium further comprises 1 to 100 micromolar biotin.

25. The method of claim 19, wherein said adipocyte medium comprises 1 to 100 micromolar pantothenate.

26. The method of claim 19, wherein the said cyclic AMP inducer comprises 0.5 to 5 mM isobutylmethylxanthine.

27. The method of claim 19, wherein the said differentiation medium and said adipocyte medium contains 100 nM human insulin.

28. The method of claim 19, wherein the said fetal serum is fetal bovine serum.

29. The method of claim 19, wherein the concentration of said glucocorticoid is 10 nM to 1 micromolar.

30. The method of claim 19, wherein said PPAR gamma agonist is 10 nM to 100 micromolar of a thiazolidinedione.

31. The three dimensional biomaterial containing adipose tissue-derived stromal cells capable of generating an adipose tissue depot upon implantation into a host recipient prepared according to the method of claims 19-30.

32. A method for grafting adipose tissue into a subject, comprising: introducing the biomaterial of claim 31 into said subject.

33. The method of claim 32, wherein said biomaterial is introduced by subcutaneous implantation.

34. The biomaterial of claim 31, wherein the cells in said three dimensional matrix are labeled with a detectable probe.

35. The method of claim 19, wherein said matrix is a poly lactic-co-glycolic disks having a porosity of 50 to 95%.

36. The method of claim 35, wherein said porosity is 90%.

37. The method of claim 35, wherein the thickness of said disk is 1 to 25 mm.

38. The method of claim 37, wherein said thickness is about 2.5 to 12.5 mm.

39. The method of claim 35, wherein said disks has a pore size of 50 to 1000 micrometers.

40. The method of claim 39, wherein said pore size is 200 to 600 micrometers.

41. The method of claim 19, wherein said matrix comprises Matrigel.

HUMAN CULTURED PREADIPOCYTES AND HUMAN CULTURED DIFFERENTIATED ADIPOCYTES.

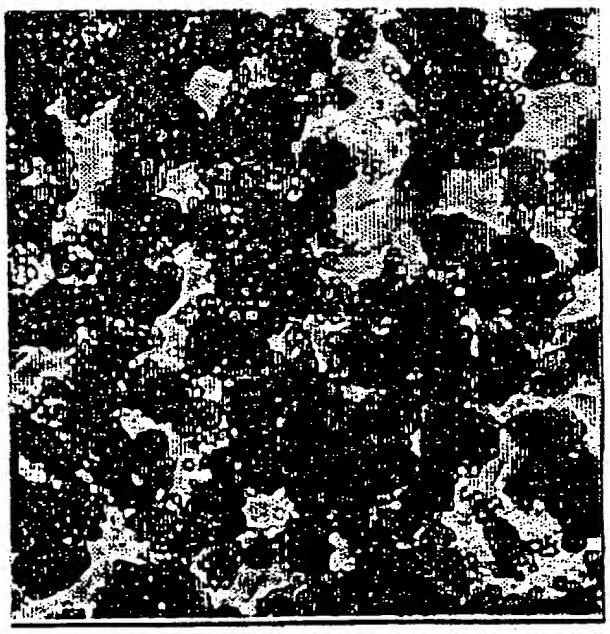


FIG. 1B.

FIG. 1A.

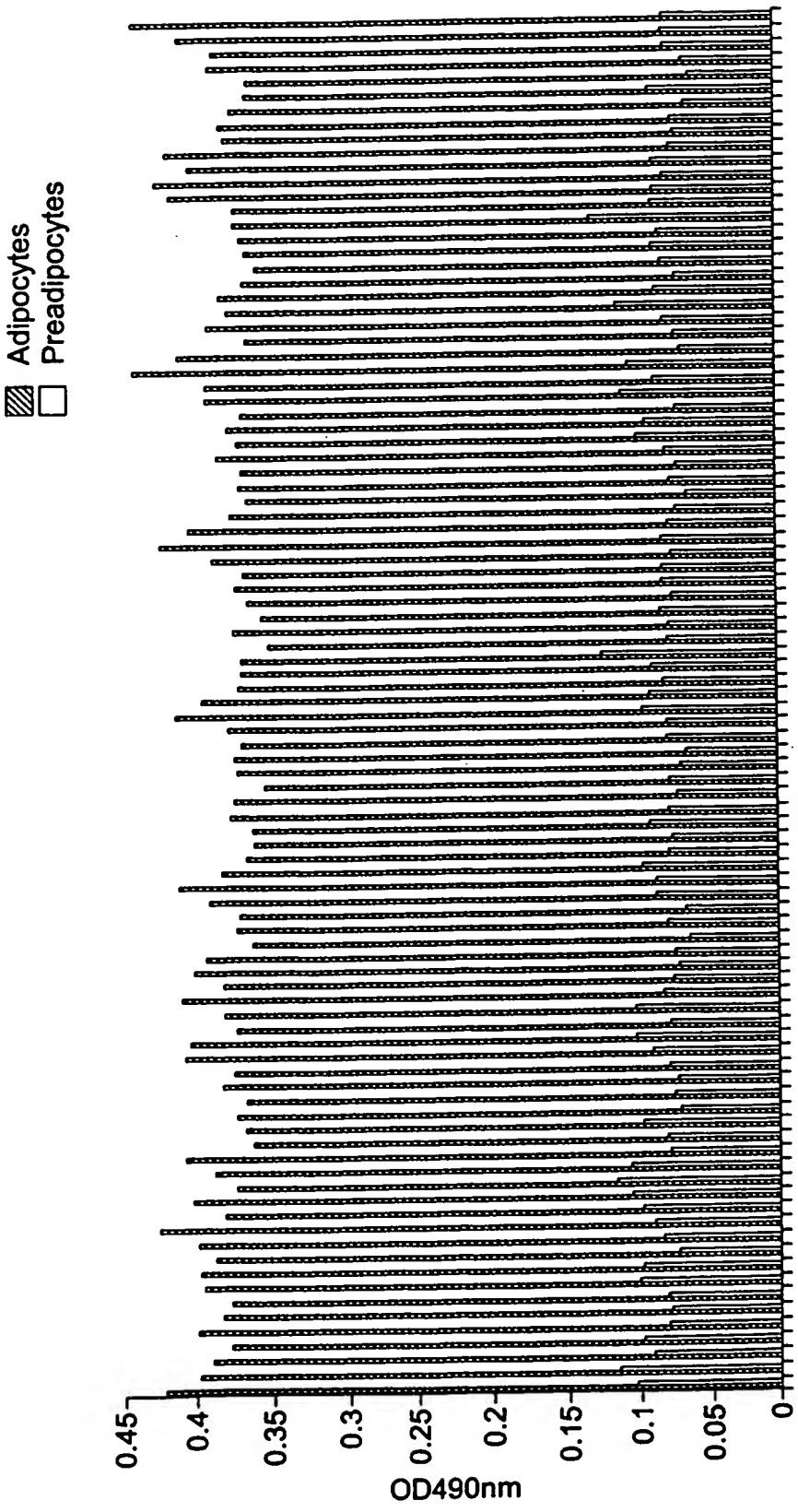

Adipocytes
Preadipocytes
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
OD490nm
WELL NUMBER

FIG. 2.

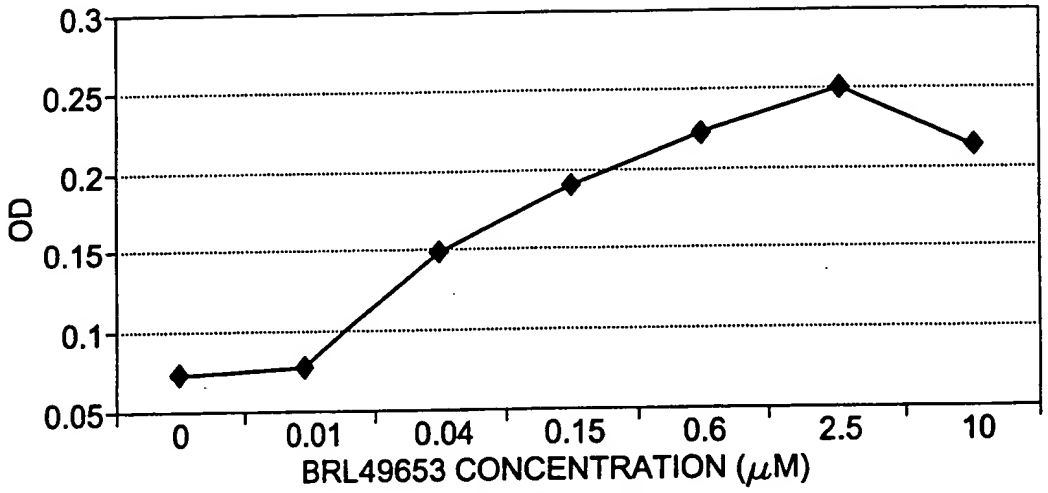

0.3
0.25
0.2
0.15
0.1
0.05
OD
0
0.01
0.04
0.15
0.6
2.5
10
BRL49653 CONCENTRATION (μM)

FIG. 3.

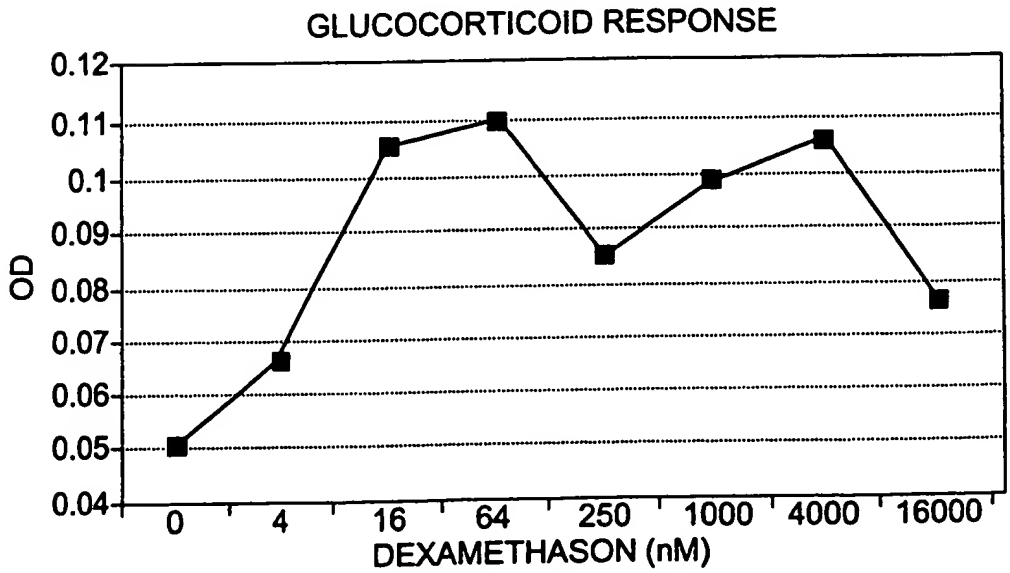

GLUCOCORTICOID RESPONSE
0.12
0.11
0.1
0.09
0.08
0.07
0.06
0.05
0.04
OD
0
4
16
64
250
1000
4000
16000
DEXAMETHASON (nM)

FIG. 4.

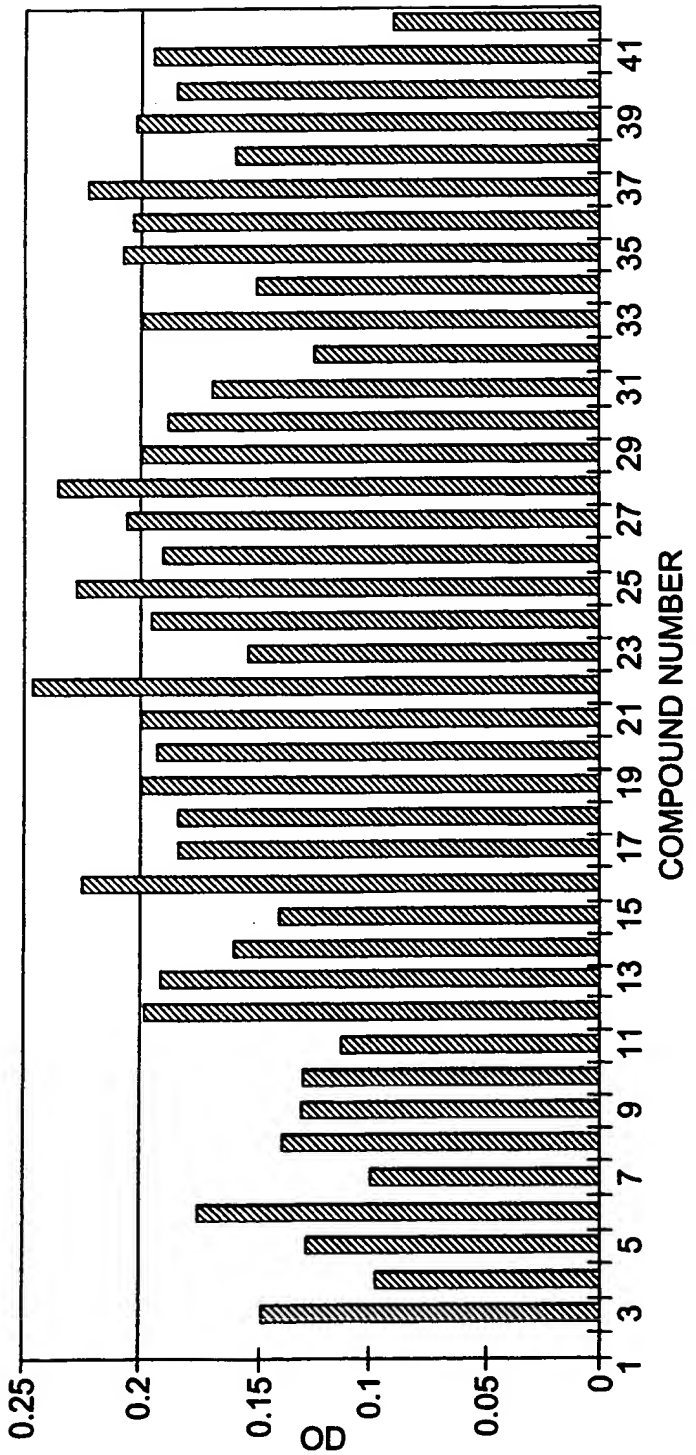

0.25
0.2
0.15
0.1
0.05
0
OD
1
3
5
7
9
11
13
15
17
19
21
23
25
27
29
31
33
35
37
39
41
COMPOUND NUMBER

FIG. 5.

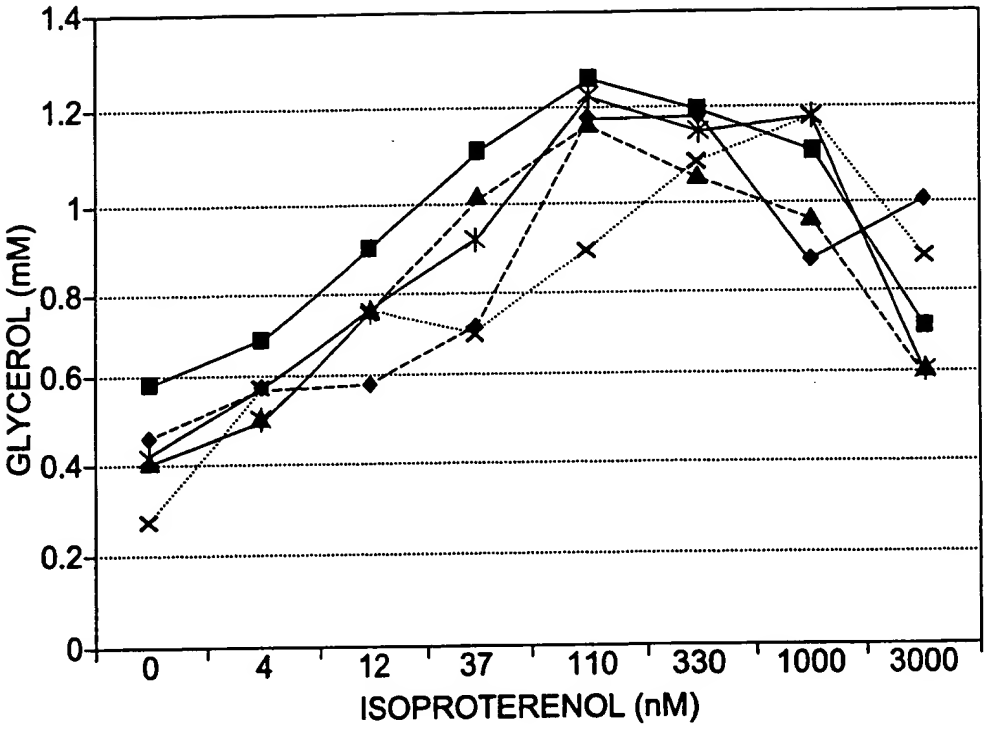

GLYCEROL (mM)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
4
12
37
110
330
1000
3000
ISOPROTERENOL (nM)

FIG. 6.

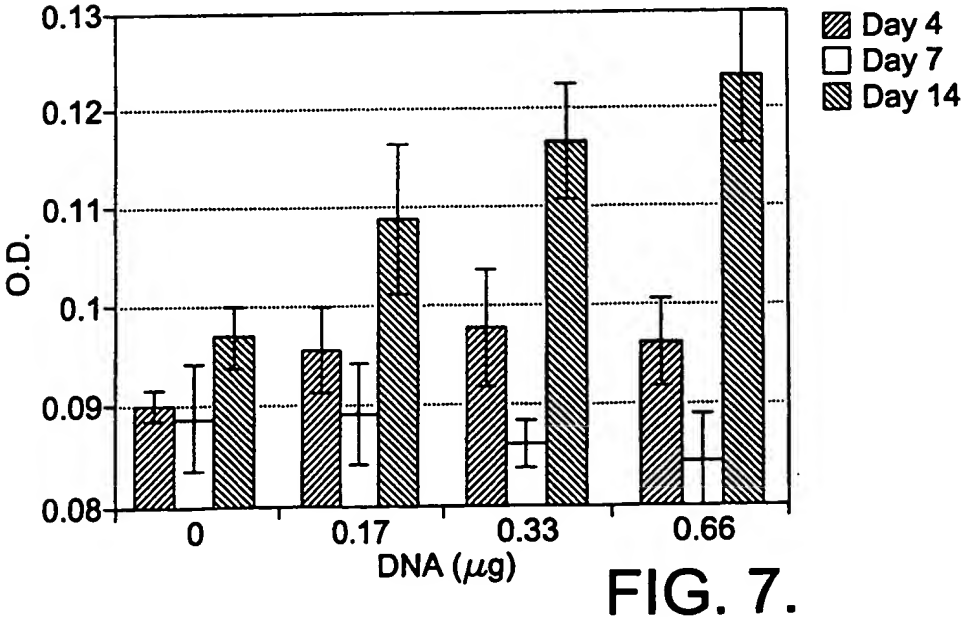

O.D.
0.13
0.12
0.11
0.1
0.09
0.08
0
0.17
0.33
0.66
DNA (μg)
Day 4
Day 7
Day 14

FIG. 7.

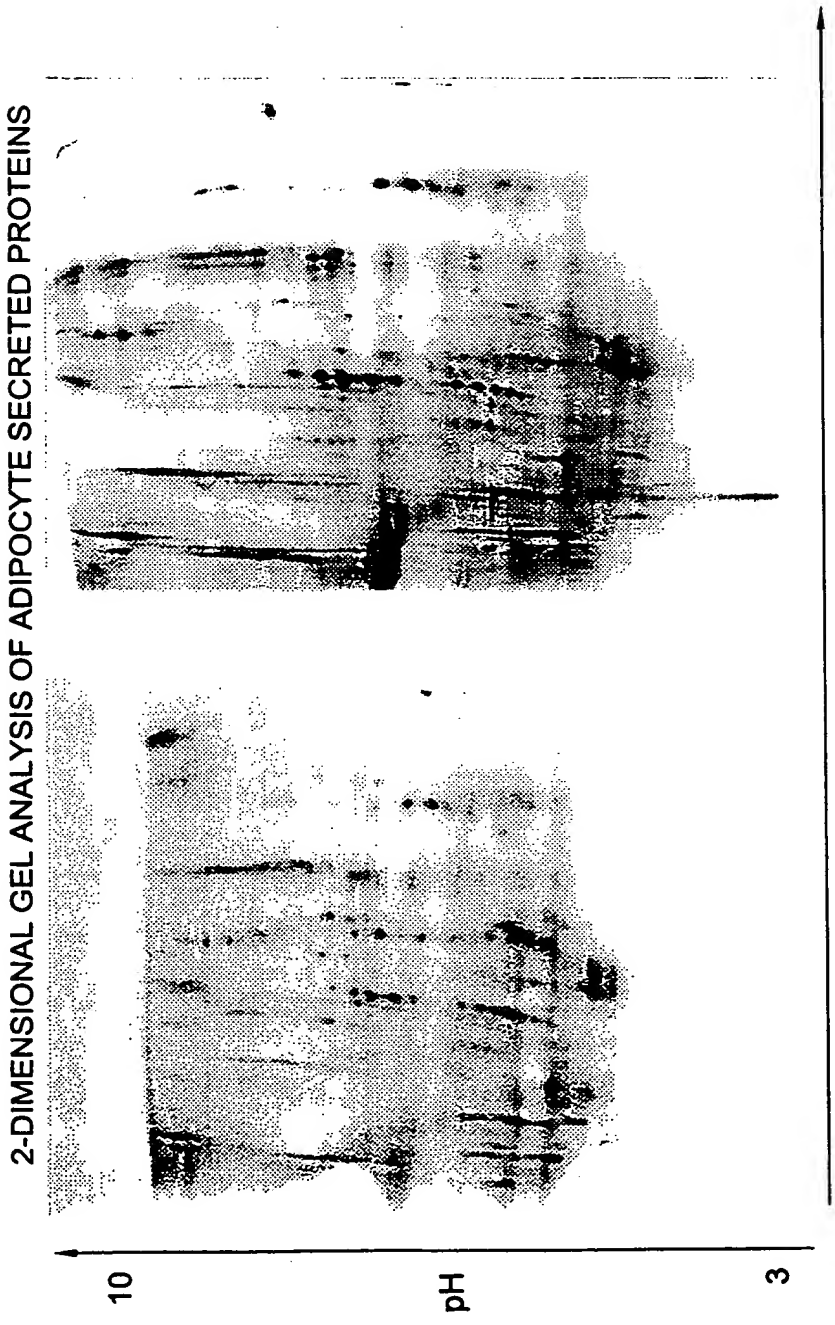

2-DIMENSIONAL GEL ANALYSIS OF ADIPOCYTE SECRETED PROTEINS
ADIPOCYTE
PREADIPOCYTE
MW
pH
10
3

FIG. 8.

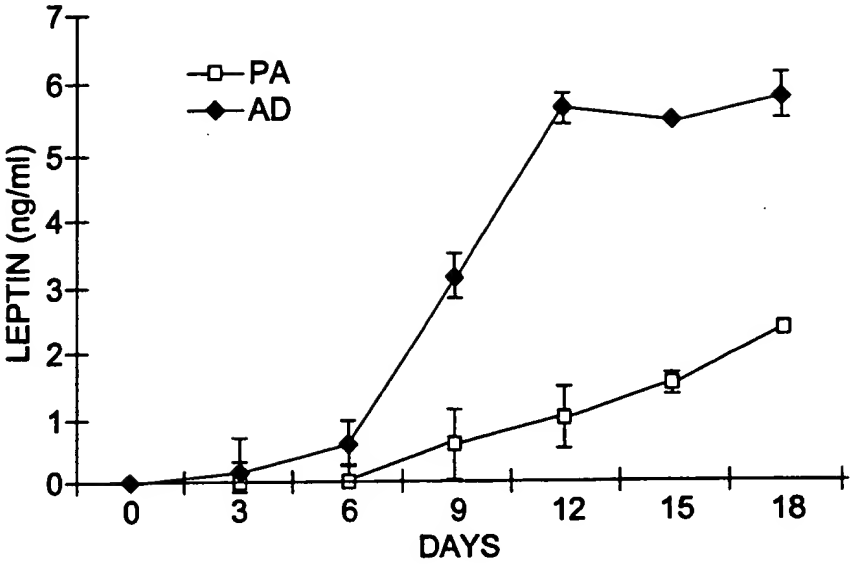

7
6
5
4
3
2
1
0
LEPTIN (ng/ml)
PA
AD
0
3
6
9
12
15
18
DAYS

FIG. 9A.

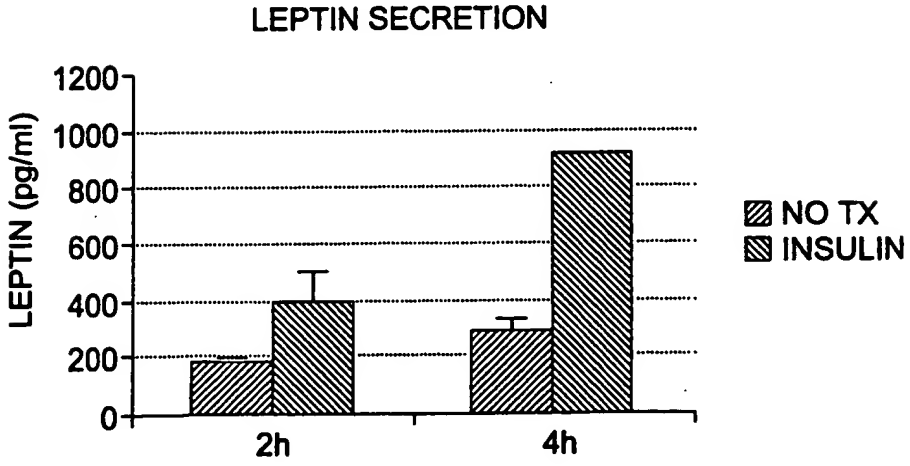
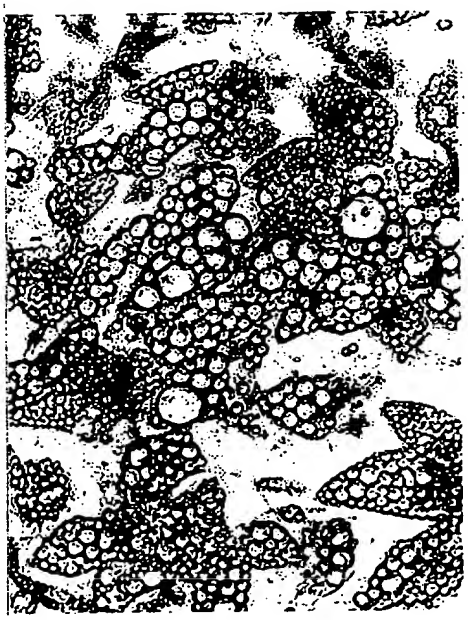

LEPTIN SECRETION
1200
1000
800
600
400
200
0
LEPTIN (pg/ml)
2h
4h
NO TX
INSULIN

FIG. 9B.

adipocytes infected with control adenoviral vectors

FIG. 10B.



ADIPOCYTES INFECTED WITH β-GALACTOSIDASE EXPRESSING ADENOVIRAL VECTORS

FIG. 10A.